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(54) Title: ACTIVITY-DEPENDENT CYSTEINE PROTEASE PROFILING REAGENT

(57) Abstract: Probes are provided having specificity for papain cysteine hydrolases comprising an electrophile, exemplified by an epoxide, a hydrophobic group for fitting into the hydrolase pocket and a moiety that provides for detection and/or isolation. A variety of compound having hydrophobic side chains form an oligopeptide are exemplified using fluorescers, ligand members of specific binding pairs or radioactive labels for detection and/or isolation.

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ACTIVITY-DEPENDENT CYSTEINE PROTEASE PROFILING REAGENT

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to USSN 60/266,295, filed on November 5, 2000, to USSN 60/287,993, filed on May 1, 2001, and to USSN 60/308,905, filed on July 30, 2001, and to USSN 60/315,117, filed on August 27, 2001, all of which are incorporated herein by reference in their entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

10 [Not Applicable]

FIELD OF THE INVENTION

[0002] This invention pertains to the field of proteomics. In particular, this invention provides novel probes that are useful for profiling cysteine hydrolase activity, for screening for selective inhibitors of various cysteine hydrolases, and for inhibiting 15 various cysteine hydrolases.

BACKGROUND OF THE INVENTION

[0003] Various approaches for studying global cellular processes permit the analysis of differential changes within large sets of known and unknown genes or proteins. DNA microarray techniques allow analysis of genome-wide changes in mRNA 20 transcription for a given cellular stimulus (Schena *et al.* (1998) *Trends in Biotechnology* 16: 301-306; DeRisi and Iyer (1999) *Curr. Opin. Oncol.* 11: 76-79. Advances in 2D gel electrophoresis coupled to highly sensitive mass spectrometry techniques now allow the rapid identification of proteins from whole cells or tissue extracts (Jungblut *et al.* (1999) *Electrophoresis* 20: 2100-2110; Celis *et al.* (1998) *Febs Letts.*, 430: 64-72). While these 25 techniques have revolutionized the global analysis of biological processes, often information about function of enzymatic proteins can only be inferred by analysis of transcriptional/translational co-regulation of sets of genes under different stimuli. However, levels of transcription and translation of an enzyme, in many cases, do not

correlate with its activity (Gygi *et al.* (1999) *Molecular and Cellular Biology* 19: 1720-1730).

5 [0004] To assign function to enzymatic proteins on a genome-wide scale, a method to obtain direct information about enzymatic activity is necessary. Since the simultaneous targeting of all enzyme classes with a single probe is likely to be impossible, the present invention focuses on typically proteolytic enzymes, in particular the cysteine hydrolases.

10 [0005] In particular, the papaine family of cysteine proteases serves as a good model system for several reasons. Firstly, most cysteine proteases are synthesized with an inhibitory propeptide that must be proteolytically removed to activate the enzyme (Cygler, *et al.* (1996) *Structure* 4: 405-416; Coulombe *et al.* (1996) *EMBO J.* 15: 5492-503) resulting in expression profiles that do not directly correlate with activity. Secondly, the largest set of papaine-like cysteine proteases, the cathepsins, act in concert to digest a protein substrate. Thus, information regarding regulation of activity of each member relative to one another is critical for understanding their collective function. Furthermore, 15 the cathepsins are involved in many critical biological processes, and biochemical studies of function have been limited to family members that have been cloned and expressed or purified from crude tissue. Finally, a large body of information is available regarding covalent, suicide substrate inhibitors that specifically target this family of cysteine proteases.

20 [0006] The papaine family is classified into several major groups, most notable of which are the bleomycin hydrolases, calpains, caspases, and cathepsins. To date, 14 human cathepsins have been cloned and sequenced. Several of these proteases are key players in normal physiological processes such as antigen presentation (Villadangos *et al.* (1999) *Immun. Rev.*, 172: 109-120), bone remodeling (Gelb *et al.* (1996) *Science* 273: 1236-1238) and prohormone processing (Beinfeld (1998) *Endocrine* 8: 1-5). In addition, 25 several of these proteases are involved in pathological processes such as rheumatoid arthritis (Iwata *et al.* (1997) *Arthritis and Rheumatism*, 40: 499-509), cancer invasion and metastasis (Yan *et al.* (1998) *Biol. Chem.*, 379: 113-123) and Alzheimer's disease (Golde *et al.* (1992) [see comments] *Science* 255: 728-730; Munger *et al.* (1995) *Biochem. J.*, 311: 299-305).

[0007] The enzymatic mechanism used by the papaine family of proteases has been well studied and is highly conserved. Thus, electrophilic substrate analogs that are only reactive in the context of this conserved active site can be used as general probes of function. A wide range of electrophiles have been developed as mechanism-based, 5 cysteine protease inhibitors including diazomethyl ketones (Shaw, E. (1994) *Meth. Enzym.*, 244: 649-656), fluoromethyl ketones (Shaw *et al.* (1986) *Biomedica Biochimica Acta* 45: 1397-1403), acyloxymethyl ketones (Pliura *et al.* (1992) *Biochem. J.*, 288: 759-762), O-acylhydroxylamines (Brömmle *et al.* (1989) *Biochem. J.*, 263: 861-866), vinyl sulfones (Palmer *et al.* (1995) *J. Med. Chem.*, 38: 3193-3196), and epoxysuccinic 10 derivatives (Barrett and Hanada (1982) *Biochem. J.*, 201: 189-198). These inhibitors typically consist of a peptide specificity determinant attached to an electrophile that becomes irreversibly alkylated when bound in close proximity to an attacking nucleophile.

[0008] Several groups have recognized the value of using irreversible mechanism-based inhibitors as affinity labels (Rauber *et al.* (1988) *Analyt. Biochem.*, 168: 259-264; 15 Bogyo *et al.* (1998) *Chem Biol.*, 5: 307-320; Bogyo *et al.* (2000) *Chem Biol.*, 7: 27-38; Mason *et al.* (1989) *Biochem. J.* 257: 125-129; Mason *et al.* (1989) *Biochem. J.* 263: 945-949).

[0009] Similar affinity labeling approaches have been used extensively to study or identify proteases such as the proteasome (Bogyo *et al.* (1998) *Chem Biol.*, 5: 307-320; 20 Bogyo *et al.* (1997) *Proc. Natl. Acad. Sci., USA*, 94: 6629-6634; Meng *et al.* (1999) *Proc. Natl. Acad. Sci., USA*, 96: 10403-10408), caspases (Faleiro *et al.* (1997) *EMBO J.* 16: 2271-2281; Nicholson and Lazebnik (1995) *Nature* 376: 37-43), cathepsins (Bogyo *et al.* (2000) *Chem Biol.*, 7: 27-38; Mason *et al.* (1989) *Biochem. J.* 263: 945-949), and 25 methionine amino peptidase (Griffith and Liu (1997) *Chem Biol.*, 4: 461-471; Sin, *et al.* (1997) *Proc. Natl. Acad. Sci., USA*, 94: 6099-6103). Cravatt and co-workers have taken advantage of the broad class-specific reactivity of fluorophosphonates towards serine proteases (Liu *et al.* (1999) *Proc. Natl. Acad. Sci., USA*, 96: 14694-14699). By incorporation of a simple, extended alkyl chain capped with a biotin moiety, they have created a broad serine protease-specific probe (FP-Biotin) for functional proteomic 30 analysis of serine proteases in cells and/or crude cellular extracts.

[0010] There is interest in developing specific compounds that have narrow or broad range specificity for target cysteine enzymes. These compounds can not only serve

to identify their target enzymes in cells, particularly where the cells are associated with particular indications, but may also serve to map the surface of the target site of the target enzymes, where variations in structure and polarity can serve to develop compounds that may serve as reversible or irreversible inhibitors of the target enzymes.

5

SUMMARY OF THE INVENTION

[0011] This invention provides functional proteomics tools that can be used to determine global patterns of activity for cysteine proteases, especially the papain family of cysteine proteases. In particular, compounds (e.g., probes) that specifically bind to cysteine proteases are provided. Preferred compounds of this invention comprise a specificity determining group bound to electrophile active group that reacts at the active site of the target enzyme (e.g. cysteine hydrolase). Preferred compounds additionally comprise a group that imparts a desirable functionality (e.g. a detectable signal) to the compound.

[0012] Particularly preferred, probes comprising epoxides, usually of a defined stereochemistry, are employed linked to a hydrophobic moiety that fits into or otherwise interacts with the active site of the target cysteine protease. Contact of the probe to the target cysteine protease results in covalent bonding of the probe to the enzyme. A variety of different hydrophobic groups are found to vary the specificity and the particular enzyme to which the probe binds. Certain preferred compounds (probes) of this invention are illustrated herein by formulas I-XI.

[0013] In certain embodiments, this invention expressly excludes DCG-04 and/or DCG-03. In addition, or alternatively, this invention can expressly exclude all (e.g., 19) members of the probe library described in Example 1. In certain embodiments, the invention also expressly excludes any one or more or all probes described in Greenbaum *et al.* (2000) *Chem. Biol.*, 7(8): 569-581; Shaw (1994) *Meth. Enzym.*, 244: 649-656, Shaw *et al.* (1986). *Biomedica Biochimica Acta* 45: 1397-1403, Pliura *et al.* (1992) *Biochem. J.*, 288: 759-762, Brömme *et al.* (1989) *Biochem. J.*, 263: 861-866, Palmer *et al.* (1995) *J. Med. Chem.*, 38: 3193-3196, Barrett and Hanada (1982) *Biochem. J.*, 201: 189-198, Rauber *et al.* (1988) *Analyt. Biochem.*, 168: 259-264; Bogyo *et al.* (1998) *Chem Biol.*, 5: 307-320; Bogyo *et al.* (2000) *Chem Biol.*, 7: 27-38; Mason *et al.* (1989) *Biochem. J.* 257: 125-129; Mason *et al.* (1989) *Biochem. J.* 263: 945-949, Bogyo *et al.* (1997) *Proc. Natl.*

Acad. Sci., USA, 94: 6629-6634; Meng *et al.* (1999) *Proc. Natl. Acad. Sci., USA*, 96: 10403-10408, Faleiro *et al.* (1997) *EMBO J.* 16: 2271-2281; Nicholson and Lazebnik (1995) *Nature* 376: 37-43; Bogyo *et al.* (2000) *Chem Biol.*, 7: 27-38; Mason *et al.* (1989) *Biochem. J.* 263: 945-949; Griffith and Liu (1997) *Chem Biol.*, 4: 461-471; Sin, *et al.* (1997) *Proc. Natl. Acad. Sci., USA*, 94: 6099-6103; Liu *et al.* (1999) *Proc. Natl. Acad. Sci., USA*, 96: 14694-14699; and/or Hawthorne *et al.* (1998) *Anal. Biochem.*, 261: 131-138.

5 [0014] The compounds of this invention to provide means for profiling cells for the active cysteine proteases being expressed, and means to screen for and/or to design
10 specific drugs as inhibitors. The compounds of this invention can be used with combinatorial libraries may be used to compounds with different specificities for various target cysteine proteases.

15 [0015] These compounds of this invention provide functional information that can be used in concert with existing genomic and proteomic methods to correlate gene and protein expression profiles with enzymatic activity. Furthermore, diversification of core compounds using solid-phase combinatorial chemistry provides libraries of compounds that can be used to obtain information about inhibitor specificities of targeted protease. This information is of use in the generation of selective inhibitors without the need for prior characterization and purification of protease targets. Addition of a reporter function, 20 such as a radioactive iodine, to inhibitors permits the visualization of covalently modified proteases in a standard SDS-PAGE gel format. Labeling intensity provides a read-out of relative enzymatic activity. Furthermore, both known and novel proteases are targets for analysis by this methodology.

DEFINITIONS

25 [0016] The terms "polypeptide", "oligopeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The term also includes variants on the traditional peptide 30 linkage joining the amino acids making up the polypeptide.

[0017] The term "residue" or "amino acid" as used herein The term "residue" as used herein refers to natural, synthetic, or modified amino acids. Various amino acid analogues include, but are not limited to, 2-amino adipic acid, 3-amino adipic acid, beta-alanine, beta-aminopropionic acid, 2-aminobutyric acid, 4-aminobutyric acid, piperidinic acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisobutyric acid, 2-aminopimelic acid, 2,4-diaminobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, hydroxylysine, allo-hydroxylysine, 3-hydroxyproline, 4-hydroxyproline, isodesmosine, allo-isoleucine, N-methylglycine, sarcosine, N-methylisoleucine, 6-N-methyllysine, 10 norvaline, norleucine, ornithine, etc.

[0018] The term "cysteine hydrolases" is used herein consistently with conventional usage of those of skill in the art. The family of cysteine proteases is characterized in a number of publications known to those of skill in the art (see, e.g., Rawlings and Barrett, (1994) *Meth. Enzymology*, 224: 461-486, Academic Press, S.D.).

[0019] The "papaine protease family" refers to a family of serine hydrolases based on structural homology to enzymes including papaine.

[0020] As used herein, an "antibody" refers to a protein or glycoprotein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively.

[0021] Antibodies exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for

example, pepsin digests an antibody below (*i.e.* toward the Fc domain) the disulfide linkages in the hinge region to produce F(ab)'2, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)'2 may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the (Fab')2 dimer into an Fab' monomer. The Fab' monomer is essentially a Fab with part of the hinge region (see, Paul (1993) *Fundamental Immunology*, Raven Press, N.Y. for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically, by utilizing recombinant DNA methodology, or by "phage display" methods (see, *e.g.*, Vaughan *et al.* (1996) *Nature Biotechnology*, 14(3): 309-314, and PCT/US96/10287). Preferred antibodies include single chain antibodies, *e.g.*, single chain Fv (scFv) antibodies in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide.

15 [0022] The term "probe" refers to a molecule that specifically binds to a target molecule (preferably a cysteine hydrolase) and provides a detectable signal or tag that can be used to detect and/or quantify the target molecule. The term probe can also refer to the probe molecule in combination with other reagents, *e.g.* a buffer system. The particular usage will be clear from the context.

20 [0023] A "probe library" refers to a collection of different probes, preferably a collection of different probes having the structure represented in formula I. The probe library comprises at least 2, preferably at least 4, more preferably at least 10, most preferably at least 19 different probes. Certain larger libraries comprise at least 20, preferably at least 50, more preferably at least 100, and most preferably at least 1000, or at least 4,000 different probes.

25 [0024] The phrase "modulate the activity" when used in reference to an enzyme (*e.g.* a cysteine hydrolase) refers to increasing or decreasing the activity of the enzyme. The increase or decrease can be effected by direct interactions between the enzyme and a "modulating agent" and/or by indirect interactions, *e.g.* with cofactors, or other components in a pathway that effects activity of the enzyme. The increase or decrease can also be by an increase or decrease in transcription and/or translation of the enzyme.

[0025] An "electrophile" refers to a chemical compound or group that is attracted to electrons and/or tends to accept electrons, particularly when in the presence of an "electron-rich" species.

[0026] The term "specific binding" when used with respect to a probe of this invention refers to binding of a target protein by a probe where the binding is diminished or lost when the target protein is denatured (*e.g.* heat denatured). Thus, in preferred embodiments, specific binding is a function of the secondary and/or tertiary structure of the target protein. The binding is regarded as "diminished" where there is a difference between the binding of the probe to the undenatured protein and the binding of the probe to the denatured protein is measurable, and preferably where the difference is statistically significant (*e.g.* at greater than 80%, preferably greater than about 90%, more preferably greater than about 98%, and most preferably greater than about 99% confidence level). Particularly preferred embodiments, specific binding shows a at least a 1.2 fold, preferably at least a 1.5 fold, more preferably at least a 2 fold, and most preferably at least a 4 fold or even a 10-fold difference from the denatured protein. In a most preferred embodiment, binding of the probe to a denatured protein sample is essentially indistinguishable from the background signal.

[0027] A "binding profile" or a "specificity fingerprint" is a pattern of binding of one or more probes of this invention to a biological sample or to a component of a biological sample.

[0028] The term "ligand" refers to functional group, atom, or molecule that is attached to another atom or molecule (*e.g.*, in this case the probe) that can combine with and thereby bind to another substance.

[0029] An "affinity tag" refers to a molecule or domain of a molecule that is specifically recognized and bound by another molecule (*i.e.* a cognate binding partner). Examples of affinity tags include, but are not limited to biotin, avidin, streptavidin, Ni-NTA, His₆, and the like.

[0030] An "epitope tag" refers to a molecule or domain of a molecule that is specifically recognized by an antibody. However, the term "epitope tag" can be used more broadly to also include a molecule or domain of a molecule bound by a binding partner (ligand) other than an antibody. In this instance the terms "epitope tag" and "affinity tag"

are similar. Thus, for example, in addition to epitopes recognized in epitope/antibody interactions, epitope tags can also comprise "epitopes" recognized by other binding molecules (e.g. ligands bound by receptors), ligands bound by other ligands to form heterodimers or homodimers, His₆ bound by Ni-NTA, and the like.

5 [0031] The terms "linker" and "spacer" are used interchangeably. A wide variety of linkers (spacers) are suitable for use in the probes of this invention. Such linkers include, but are not limited to straight or branched-chain carbon linkers, heterocyclic carbon linkers, and the like. Preferred linkers are C₁ to C₂₀, more preferably C₂ to C₁₀, and most preferably C₃ to C₆ straight chain carbon linkers. Particularly preferred linkers
10 include, but are not limited to straight chain saturated alkyl amino acids such as amino hexanoic acid, as well as spacers greater or fewer methylene groups (e.g. between 2 and 10 methylene groups). The linkers can also include various cleavable linkers that can be used to selectively release probe-modified peptides. A number of different cleavable linkers are known to those of skill in the art (see, e.g., U.S. Patent Nos: 4,618,492,
15 4,542,225, and 4,625,014). The mechanisms for release of an agent from these linker groups include, for example, irradiation of a photolabile bond and acid-catalyzed hydrolysis. One particularly preferred linker is a photolabile linker (PhotoReleaseTM) that can be used to selectively release probe-modified peptides by UV irradiation. This linker is commercially available from Advanced Chemtech. Also a free amino lysine residue can
20 be used as a spacer in place of the lysine-biotin conjugate that can be covalently attached to affigel (BioRad) to create an affinity resin. The linker can also include a 1-2-diol moiety that could be cleaved by mild oxidation with sodium periodate to specifically release peptide products from affigel or from streptavidin agarose. Representative oxidizable cleavable linkers are illustrated in Figure 11 and various photolabile cleavable
25 linkers are illustrated in Figure 12.

30 [0032] The term "small organic molecule" refers to a molecule of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e.g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 5000 Da, more preferably up to 2000 Da, and most preferably up to about 1000 Da.

[0033] The term "modified streptavidin" refers to a monomeric avidin or streptavidin or to a derivatized streptavidin or to a streptavidin analog. Certain modified streptavidins show reduced affinity to biotin.

5 [0034] The term "biological sample", as used herein, refers to a sample obtained from an organism, from components (e.g., cells or tissues) of an organism, and/or from *in vitro* cell or tissue cultures. The sample may be of any biological tissue or fluid (e.g. blood, serum, lymph, cerebrospinal fluid, urine, sputum, etc.). Biological samples may also include organs or sections of tissues such as frozen sections taken for histological purposes.

10 [0035] The term "crude cellular extract" refers to a relatively unpurified or completely unpurified derivative obtained from one or more cells. A typical crude cellular extract is simply a suspension of homogenized cells. Certain crude cellular extracts include cellular extracts that have been filtered, centrifuged, or otherwise treated to remove particulate matter.

15

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] Figure 1 illustrates the structure of epoxide inhibitors and probes E-64, JPM-565 and DCG-04. Preferred radiolabel attachment and affinity sites are indicated for each compound.

20 [0037] Figures 2A and 2B illustrate the synthesis of DCG-04. Figure 2A illustrates the epoxy acid building block (epoxide (I)) and Figure 2B illustrates a solid-phase synthesis scheme for DCG-04. Details of the synthesis and characterization of peptide epoxides can be found herein in Example 1.

25 [0038] Figures 3A and 3B illustrate DCG-03 and DCG-04 labeling of active proteases in dendritic cell extracts. Figure 3A: Total cell extracts from DC2.4 cells were diluted into either pH 5.5 or pH 7.4 buffer, preheated to 100°C for 1min (+ preheating) or not (-preheating) and labeled with 50 μ M DCG-03 and DCG-04. Samples were separated by SDS-PAGE (12.5% gel) and labeled bands visualized by affinity blotting as described in the experimental section. Figure 3B: Same as for Figure 3A except 125 I labeled versions of DCG-03 and DCG-04 were used and the gels were analyzed by

autoradiography. The location of cathepsin B, L, and S are indicated for reference based on their known molecular weights.

[0039] Figures 4A and 4B show that DCG-03 and DCG-04 target the same polypeptides as the parent compounds E-64 and JPM-565. Figure 4A: Total cellular extracts from DC2.4 cells were incubated with increasing concentrations of E-64 as indicated for 30 min at 25°C followed by addition of 50 μ M DCG-04 and further incubation for 1 hr. Samples were resolved by SDS-PAGE (12.5%) and labeled bands visualized by affinity blotting. Figure 4B: Total cellular extracts were labeled with either 125 I-labeled forms (auto-rad) or with non-labeled forms (blot) of DCG-03, DCG-04, and JPM-565 followed by separation by SDS-PAGE (12.5%) and analysis as indicated. The location of cathepsin B and S are indicated for reference based on their known molecular weights.

[0040] Figures 5A and 5B illustrate activity profiling across a disease progression. Tissue culture cells were isolated from carcinomas generated by application of a chemical mutagen to the skin of mice (see Example 1). Progression begins at the left with the non invasive benign cells (C5N and P6) and progresses to the right through papilloma cell lines (PDV and PDV-C57), squamous cell carcinomas (B9, A5, and D3), and finally highly invasive spindle cell carcinomas (Car B and Car C). Total cellular lysates were normalized with respect to protein concentration and labeled with 125 I-DCG-04 (Figure 5A) and the cathepsin B-specific probe 125 I-MB-074 (Figure 5B). A pre-heat control from the C5N lysate was included in A) to show background labeling.

[0041] Figure 6 illustrates profiling protease inhibitor specificity. Lysates from the dendritic cell line DC2.4 (panels A and B) or purified cathepsin H (panel C) were preincubated with 50 μ M of each of the 19 derivatives of DCG-04 and then labeled with 125 I-DCG-04 (panels A and C) or 125 I-MB-074 (panel B) as indicated. The general structure of the inhibitors is shown with the variable amino acid sidechain indicated as an X (competitor; top). The predominant labeled polypeptides in A) are labeled with numbers and positions of cathepsin B and S are indicated for reference.

[0042] Figure 7 shows activity profiling of cysteine proteases across tissue types. Labeling of total cellular extracts (100 μ g protein/lane) from rat brain, kidney, liver, prostate, and testis with 125 I-DCG-04 at pH 5.5. Samples were analyzed by SDS-PAGE

followed by autoradiography. A pre-heating control was included for each tissue type to indicated background labeling.

[0043] Figure 8 illustrates affinity purification of DCG-04 targeted proteases from rat kidney. Panel A illustrates labeling of total cellular extracts (100 μ g protein/lane) from rat kidney with 50 μ M DCG-04 at pH 5.5. Samples were analyzed by SDS-PAGE followed by affinity blot. Panel B shows the results of anion exchange chromatography of rat kidney lysate using a gradient from 0.05-1M NaCl, pH 9.0. Fractions were analyzed by addition of DCG-04 (50 μ M) followed by SDS-PAGE and affinity blotting. Fractions containing DCG-04 labeled proteins were pooled (fractions 5-7 and Fractions 11-13).
10 Panel C: Pooled fractions were labeled with DCG-04 (50 μ M), and DCG-04 modified proteins bound to a monomeric-avidin column, washed with 1M NaCl, and eluted using 2mM biotin. A sample of material from pools prior to application to the affinity column (PC) along with column flow through (FT) and biotin elution fractions (E1-E5) were analyzed by SDS-PAGE followed by silver staining. Panel D: Elutions containing
15 labeled proteins were pooled, volumes reduced, and analyzed by 2D IEF electrophoresis followed by silver staining. Spots labeled with numbers were excised and used for sequencing.

[0044] Figure 9 shows a low energy CID spectrum of tryptic peptides with MH^+ = 1429.7. The doubly charged ion at m/z 715.35 was selected as a precursor ion. Only the 20 C-terminal fragment ions used for sequence determination are labeled.

[0045] Figure 10 shows certain preferred probes of this invention.

[0046] Figure 11 shows various cleavable (oxidizable) linkers.

[0047] Figure 12 shows various cleavable (photolabile) linkers.

[0048] Figure 13 shows structures of fluorescent DCG-04 probes. The four non-overlapping fluorescent DCG-04 analogs include BODIPY588/616-DCG-04 (Red-DCG-04), BODIPY493/503-DCG-04 (Blue-DCG-04), BODIPY530/550-DCG-04 (Green-DCG-04), and BODIPY558/568-DCG-04 (Yellow-DCG-04). These probes are synthesized from the corresponding DCG-04 free amine by reaction with the corresponding BODIPY succinamide ester. All fluorophores were purchased from Molecular Probes.
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30 [0049] Figures 14A and 14B illustrate affinity labeling of papain family proteases using fluorescent ABPs. Figure 14A: Purified cathepsins (as indicated) were diluted into

pH 5.5 buffer and labeled with 100nM Yellow-DCG-04, Red-DCG-04, Green-DCG-04 or Blue-DCG-04 for 1 hour. Samples were separated on a 15% SDS-PAGE gel and labeled bands visualized using an ABI 377 DNA sequencer as described in Example 2. Figure 14B: Total cell extracts from rat liver were diluted into pH 5.5 buffer and labeled with 5 10mM DCG-04, ¹²⁵I-DCG-04 (approx. 1 x 10⁶ CPM), or 100 nM red-, blue-, green-, and yellow-DCG-04. Samples were separated on a 15% SDS-PAGE gel and labeled bands were visualized (as indicated at bottom) by affinity blotting, autoradiography, or using a Molecular Dynamics Typhoon laser fluorescence scanner.

[0050] Figures 15A and 15B illustrate labeling of purified cathepsins with 10 fluorescently labeled probes and localization of protease activity *in situ*. Figure 15A: DC2.4 cells were grown in culture in serum-free media and treated overnight with Green-DCG-04 (1 mM final concentration) or Figure 15B: pre-treated with 10 mM of E-64 for 1 hour and then labeled with 1 mM of Green-DCG-04. Fresh media was added and cells 15 incubated for five hours to remove excess probe. Cells were visualized by fluorescence microscopy (Left panels) then collected, lysed in SDS sample buffer and analyzed by SDS-PAGE on an ABI 377 DNA sequencer (Right panels). Labeled proteases in the untreated cells are indicated with numbers. Note the complete competition of all protease species by E-64 pre-treatment.

[0051] Figures 16A, 16B, and 16C show the screening of peptide epoxide 20 positional scanning libraries (PSLs). Figure 16A: Structures of the general PSL scaffolds containing either (S,S) or (R,R) epoxides. PSLs contain a fixed P2 position (X) and P3 and P4 positions composed of an isokinetic mixture of 19 natural amino (all natural amino acids minus cysteine and methionine, plus norleucine; Mix). Figure 16 B: Colorimetric cluster display of inhibition data. PSLs were used to profile purified cysteine proteases by 25 pretreatment of samples with individual constant P2 libraries followed by labeling with ¹²⁵I-DCG-04. Labeling intensity of each target relative to the control untreated sample was used to generate percent competition values. These resulting data were clustered and visualized using programs designed for analysis of micro-array data (see Example 2). The tree structures at the top and left of the diagrams were obtained by hierarchical clustering 30 and indicate the degree of similarity as a function of the height of the lines connecting profiles. Figure 16C: Results from profiling proteases in rat liver extracts. Data was compiled and visualized as described in (Fig. 16B). Each constant non-natural amino acid

is indicated with a number corresponding to its structures listed in the supplemental materials. Constant natural amino acids are indicated using the standard one letter code (n is used for norleucine). Natural amino acids attached to the R,R epoxide are indicated with "R,R". Unknown protease bands in rat liver are numbered 1-4 and correspond to the 5 bands shown in figure 14B. The color key is shown at the bottom.

[0052] Figure 17A and 17B illustrate the profiling of changes in protease activity upon inhibitor treatment. Liver extracts (100 mg) were treated with 100 nM Red-DCG-04 or with 10mM of Ac-XX-Q-(R,R)Eps library for 30 minutes and then 100 nM Blue-DCG-04. Reactions were quenched with IEF sample buffer and equal amounts of each reaction 10 were co-loaded on a single IEF tube gel. Labeled proteins were separated on a 15% SDS PAGE and analyzed using an ABI 377 DNA sequencer. Figure 17A, bottom panel shows the red and blue channels overlaid on a single image while the top and middle panels show the individual labeling profiles. Note the loss of activity of the circled protease upon inhibitor treatment. Figure 17B: Active proteases in the liver extract were purified by a 15 single step affinity purification of DCG-04-labeled liver extract. Silver-stained spots were excised and sequenced by LC-MS-TOF CID. The silver-stained spot corresponding to the labeled protease inhibited by Ac-XX-Q-(R,R)-Eps library was identified as cathepsin B. Other papain family protease were also identified and are labeled with arrows.

[0053] Figures 18A and 18B illustrate the evaluation of specific protease inhibitors 20 selected from library screening. Competition analysis of a negative control compound (YG(R,R)Eps), a cathepsin B-specific compound identified from the library screening (YQ-(R,R)Eps), and a previously described cathepsin B-specific inhibitor (MB-074). Several concentrations of each compound were incubated with 100 mg total liver extract 25 for 30 minutes followed by labeling with ¹²⁵I-DCG-04 for 1 hour. (Figure 18A: Inhibition dose response profiles for each compound. Figure 18B: Direct labeling of 100 mg total liver extract with radioiodinated versions of DCG-04, MB-074 and YQ-(R,R)Eps. Note the specificity of MB-074 and YQ-(R,R)Eps for cathepsin B.

[0054] Figure 19 illustrates screening of small molecule libraries against the 30 complete set of papain family cysteine proteases in Rat liver. This image shows a typical gel image generated from scanning of the gel as well as the process by which labeled bands can be quantitated (panel to left). Small molecules can be analyzed for their potency and selectivity for targets in the rat liver proteasome using this method. Note that

each color data can be separately extracted due to non-overlapping emission spectra of the chosen fluorophores. This approach therefore allows analysis of up to 80 samples in a single gel using four color labels.

DETAILED DESCRIPTION

5 [0055] Analysis of global changes in gene transcription and translation by systems-based genomics and proteomics approaches provides only indirect information about protein function. In many cases enzymatic activity fails to correlate with transcription or translation levels. Therefore, a direct method for broadly determining activities of an entire class of enzymes on a genome-wide scale is of great utility.

10 [0056] This invention provides a class of compounds that are useful functional proteomics tools. The compounds are generally specific for cysteine proteases and can be used to determine patterns of activity for cysteine hydrolases (*e.g.* the papaine family of cysteine proteases). These compounds provide functional information that can be used in concert with existing genomic and proteomic methods to correlate gene and protein expression profiles with enzymatic activity. Furthermore, diversification of the compound specificity determinants, *e.g.*, using solid-phase combinatorial chemistry provides libraries of compounds that can be used to obtain information about inhibitor specificities of targeted cysteine hydrolases. This information is of use in the generation of selective inhibitors without the need for prior characterization and purification of

15 hydrolase/protease targets.

[0057] The compounds can be used to specifically bind to and thereby identify cysteine protease activity even in a complex biological mixture, such as a cellular cytosol or lysate.

[0058] The compounds of this invention bind to and thereby covalently modify their target protease(s). They can be used to rapidly identify and/or isolate targets (*e.g.* novel proteases). Other uses of the compounds of this invention include, but are not limited to the profiling of cysteine hydrolase activity in disease states, the analysis of selectivity of various small molecules and drugs, and the diagnostic tracking of cysteine proteases in various biological samples (*e.g.* whole cells, cell lysates, biological fluids, and

20 biopsied tissue samples).

I. Probe structure.

[0059] In preferred embodiments, the compounds of this invention comprise reactive electrophiles joined to a hydrophobic moiety that provides affinity/specificity for individual cysteine proteases or ranges (classes) of cysteine proteases. Besides the two elements of the compounds indicated above, other groups may be added to the hydrophobic moiety without interference with the specificity, while providing for other attributes, such as identification, isolation, solubility, interaction with other compounds, etc.

[0060] Besides the two elements of the compounds indicated above, other groups may be added to the hydrophobic moiety without interference with the specificity, while providing for other attributes, such as identification, isolation, solubility, interaction with other compounds, etc.

[0061] Depending upon the intended use of the compounds of this invention, the compounds can be non-labeled, labeled with a detectable label, tagged with a ligand for which a binding partner is available, joined to an effector that provides a particular enzymatic and/or catalytic activity, and the like. In this way, depending on the tag, label, effector, etc., one can detect the presence of the compound at a site (e.g. in a cell), isolate the compound, separate the reaction product of the compounds, deliver a particular catalytic or enzymatic activity to a particular location in a cell, and the like.

[0062] In certain preferred embodiments, the compounds or probes of this invention will have the following formula:

[0063] $A - L^1 - Hy - L^2 - E$

I.

[0064] where:

[0065] A can be any group, usually of at least about 15 Dal and usually not more than about 2 kDal, more usually not more than about 1 kDal, that does not interfere with the bonding of the compound to the target cysteine proteases and that imparts a desirable function to the compound (e.g. a detectable label, a ligand, etc.);

[0066] L^1 and L^2 can be the same or different and each can be a bond, a chain of from 1 to 40, usually 1 to 30 atoms, and will usually have from 0 to 36, more usually from

0 to 30 carbon atoms and from 0 to 12, usually from 0 to 8 heteroatoms, that are nitrogen, oxygen, phosphorous and sulfur, being amines, carboxy derivatives, such as amides and esters, ethers (including thioethers), and the like;

[0067] Hy is a hydrophobic group that binds with specificity to the binding site of the cysteine protease, preferably to the S2 pocket of the cysteine hydrolase, providing specificity to the compound for bonding to cysteine proteases, the hydrophobic group varying with the range of specificity desired for the compound, where the hydrophobic group will usually be of at least about 5 carbon atoms, usually at least about 6 carbon atoms and not more than about 50 carbon atoms, usually not more than about 36 carbon atoms, and may be aliphatic, alicyclic, aromatic or heterocyclic, or combinations thereof, and will have from 1 to 6, usually 1 to 4 heteroatoms, that are oxygen, nitrogen, sulfur, halogen, phosphorous, etc.; and

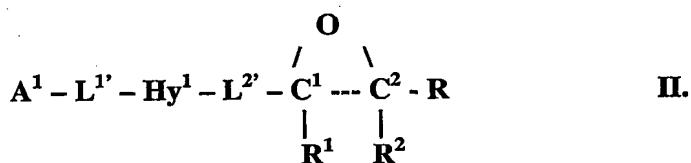
[0068] E is an electrophile that is active at the active site of the cysteine hydrolase to form a covalent bond at the . In certain preferred embodiments, the electrophile is one that is typically inert, but becomes reactive when around electron-rich species (e.g. when localized in or near the binding site of a cysteine hydrolase). In particularly preferred embodiments, electrophile includes, but is not limited to various ketones (e.g. diazomethyl ketone, fluoromethyl ketone, acyloxymethyl ketone, chloromethyl ketone, etc.), epoxides, particularly carboxy- substituted epoxides, reactive α -substituted methyl keto carbonyls, e.g. halo, diazo, and acyloxy, vinyl sulfones, O-acyl hydroxylamines, etc. In certain embodiments, E will comprise at least 2 carbon atoms and not more than about 12 carbon atoms, usually not more than about 8 carbon atoms. In certain embodiments, E will comprise at least one heteroatom and usually not more than 6 heteroatoms, where preferred heteroatoms include nitrogen, oxygen, sulfur and phosphorous.

[0069] Particularly preferred electrophiles, when coupled to the peptide specificity determinant (e.g. the hydrophobic group), form a "suicide substrate", that is, a substrate that binds essentially irreversibly with its "target" cysteine protease (e.g. by forming a covalent linkage to the target). The suitability of various electrophiles can therefore be readily assayed by coupling the electrophile to a particular substrate (e.g. -Tyr-Lys-), contacting that substrate to a target protease (e.g. a cathepsin), and determining whether the substrate/electrophile tightly (e.g. irreversibly) binds to the target protease.

[0070] A certain preferred electrophiles is an epoxide, particularly an epoxide having an activating group bonded to an annular carbon atom, particularly a carbonyl and more particularly a carboxy carbonyl. Preferably, both annular carbon atoms have activating groups. Where both annular carbon atoms are substituted, enantiomerically enhanced compositions will preferably be employed, such as R,R and S,S, substantially free of the other stereoisomer.

5 [0071] Compounds of this invention of particular interest have the following formula:

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[0072] wherein:

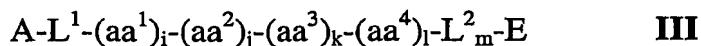
[0073] A¹ is preferably moiety of from 1 to 30, usually from 4 to 20 carbon atoms and from 0 to 10, usually 0 to 8 heteroatoms, which include N, O, S, P and halo, that provides a detectable signal, e.g. a fluorescer, or a ligand for binding to a specific receptor or other cognate binding partner, where the complex of ligand and receptor allows for specific isolation, e.g. the ligand may be referred to as an affinity tag, or binding to another molecule of interest, e.g. an enzyme or functionalized protein; wherein when said moiety provides a detectable signal, said moiety will be carbocyclic or heterocyclic aromatic, generally having rings of from 5 to 7 annular atoms, where the rings may be fused or non-fused, and may be connected by a bond or chain of from 1 to 8 atoms, which may be saturated or unsaturated, generally the unsaturation will be ethylenic unsaturation;

20 [0074] L¹ and L² (e.g. linkers) are the same or different and are preferably an aliphatic chain of from 1 to 8, usually 1 to 6 carbon atoms joined to A¹ or the epoxide C¹ annular carbon atom and Hy¹ through the same or different functional group, which can be 25 amino, amide, ester or ether (including thioether), where the chain may be substituted or unsubstituted, the total number of carbon atoms preferably being not more than 12 and there preferably being from 0 to 4 heteroatoms as described for L;

[0075] Hy^1 is a neutral, preferably hydrophobic, amino acid. Particularly preferred amino acids have at least 4, more preferably at least 5, still more preferably at least 6 carbon atoms, and generally not more than about 20 carbon atoms, usually not more than about 16 carbon atoms, that can be aliphatic, alicyclic, aromatic, or heterocyclic, branched or unbranched, aliphatically saturated or unsaturated, usually having not more than about 2 sites of unsaturation, ethylenic or acetylenic, where substituents on rings can be separated by 2, 3 or 4 annular members, the substituents normally being aliphatic groups of from 1 to 6 carbon atoms, halogen or nitrogen containing substituents, such as amino, including mono- and di-lower alkyl amino (lower alkyl is preferably of from 1 to 6, more preferably 1 to 3 carbon atoms), cyano, nitro, carboxamide, phosphoramido, and the like, there being from 1 to 3 rings, where the rings can be fused or unfused, and, when unfused, are usually separated by from 0 to 3 atoms, that is bonded together or having a bridge that will usually be alkylene, oxoalkylene, oxyalkylene, and the like; desirably there will be a side chain as the D or L stereoisomer;

[0076] the R groups are the same or different, in preferred embodiments there being not more than two of the R groups other than hydrogen, where the total number of carbon atoms for all of the R groups is from 0 to 8, usually from about 0 to 4; the R groups can include hydrogen, lower alkyl, *e.g.* of from 1 to 6, usually 1 to 3 carbon atoms, oxycarbonyl of from 1 to 3 carbon atoms, alkoxy carbonyl of from 2 to 5 carbon atoms, preferably being free of acidic groups; more preferably, R is alkoxy carbonyl and R^2 is hydrogen.

[0077] In certain preferred embodiments, the probes of this invention comprise a core amino acid or peptide recognition domain attached to the electrophile directly or through a linker (L). The probes also preferably include a ligand, affinity site or detectable label, and, in certain preferred embodiments, include a detectable label attached to the ligand or affinity site. Thus, in such particularly preferred embodiments the probes can have the formula:



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[0078] where A is a ligand, affinity tag, or detectable label, L^1 is a linker, L^2 , when present, is a linker, aa^1 , aa^2 , aa^3 , and aa^4 , when present, are independently selected amino

acids, i, j, k, l, and m are independently 0 or 1, E is an electrophile, and at least one of aa¹, aa², aa³, and aa⁴ are present.

[0079] As indicated above, "L" can be characterized as a bond or as a "linker" or "spacer" for joining the electrophile to the hydrophobic group (specificity determining group) and/or for joining the affinity tag, ligand, label, etc. to the hydrophobic group (recognition domain). Generally a spacer or linker has no specific biological activity other than to join particular components of the probe or to preserve some minimum distance or other spatial relationship between them. However, the spacer may be selected to influence some property of the probe such as the folding, net charge, or hydrophobicity 10 of the probe.

[0080] As indicated above, a wide variety of linkers (spacers) are suitable for use in the probes of this invention. Certain linkers include, but are not limited to straight or branched-chain carbon linkers, heterocyclic carbon linkers, and the like. Preferred linkers are C₁ to C₂₀, more preferably C₂ to C₁₀, and most preferably C₃ to C₆ straight chain 15 carbon linkers. In one particularly preferred embodiment the linker is a hexanoic acid linker (e.g. an amino hexanoic acid linker).

[0081] Depending upon the desired specificity of the probe Hy (aa) will vary. In preferred embodiments, it will not have acidic groups, will be free of quaternary carbon atoms, the amino group to which the linking group is linked is preferably not an annular 20 member; and the carboxy and amino are preferably not linked through a ring. Desirably, there is an aliphatic, alicyclic, aromatic side chain of from 2 to 16 carbon atoms, usually 3 to 16 carbon atoms and from 0 to 4 heteroatoms, preferably oxygen, nitrogen and sulfur, particularly at the α -carbon atom.

[0082] Hy can be a naturally occurring or unnatural amino acid, either D or L, 25 where the amino group may be α to ω , usually be from about α to δ , preferably α ; similarly the side chain may be at any site, but will come within the preferences for the amino group; usually Hy will be neutral or basic, preferably neutral, and may have amino, oxy or oxo substituents, e.g. keto and carboxy carbonyl; preferred groups include carbocyclic rings of from 5 to 7, usually 5 to 6 carbon atoms, there being from 1 to 3, usually 1 to 2 30 rings, which may be fused or unfused, aliphatic chains, branched or unbranched, saturated or unsaturated, usually having not more than 3 sites, usually not more than 2 sites of

aliphatic unsaturation, either double or triple bonds. As the groups come within the above limitations, the probes are able to react with a number of different papain cysteine hydrolases. As one deviates from the reactive moieties, greater specificity is obtained and further deviations results in specificity with lower affinity or substantially no affinity.

5 Furthermore, it appears that the R,R-stereoisomer and the S,S-stereoisomer provide for significant selectivity with the appropriate side groups.

[0083] As indicated above, suitable amino acids for incorporation into the probes of this invention include naturally occurring amino acids and modified or non-natural amino acids. Such modified amino acids include, but are not limited to, norleucine, 10 epsilon-aminocaproic acid, 4-aminobutanoic acid, tetrahydroisoquinoline-3-carboxylic acid, 8-aminocaprylic acid, 4-aminobutyric acid, α -aminoisobutyric acid, aminoisobutyric acid, aminobutyric acid, diethylglycine, α, β -dehydroaminobutyric acid, aminohexanoic acid, norvaline, τ -butylglycine, 3-cyclohexyl-alanine, phenylglycine, α -cyclohexylglycine, 3-(1-naphthyl)-alanine, 3-(2-naphthyl)-alanine, 4-(boc-amino)-phenylalanine, 15 biphenylalanine, 4-benzoyl-phenylalanine, homo-phenylalanine, α, β -dehydroleucine, α, β -dehydrovaline, 4-(aminomethyl)benzoic acid, 4-(aminomethyl) cyclohexane, 2-aminobenzoic acid, 3-aminobenzoic acid, (s)-2-amino-4-cyanobutyric acid, 4-methyl phenylalanine, p-nitrophenylalanine, pipecolic acid, isonipecotic, 1-trans-4-hydroxyproline, thiazolidine-4-carboxylic acid, (3s)-1,2,3,4-tetrahydroisoquinoline, 1-aminocyclopropane-1-carboxylic acid, 1-amino-1-cyclopentane-carboxylic acid, 1-amino-20 1-cyclohexane carboxylic acid, igl-oh, allylglycine, 3-amino-3-phenylpropionic acid, propargylglycine, (2-pyridyl)alanine, (2-furyl)-alanine, beta-styrylalanine, (2-thienyl)alanine, and the like. In certain preferred embodiments, non-natural amino acids are Fmoc-blocked.

25 [0084] Embodiments in which the peptide recognition domain of the probes of this invention is a monopeptide (*i.e.*, *i*, *j*, and *k* are zero), or a dipeptide (*i.e.* *i* and *j* are zero) show particularly good specificity for members of the papaine family of cysteine hydrolases. It is noted that, the group that binds to the S2 pocket (*i.e.* Hy) changes to this group (*e.g.* amino acid residue) are to have the greatest effect on specificity of the probes 30 of this invention for a given target (*e.g.* a particular cysteine hydrolase).

[0085] Besides the variation at the affinity site (e.g. Hy, aa₁ through aa_l), there can be variation at the other terminus of the hydrophobic moiety. As indicated previously, these can be selected for a variety of purposes. There appears to be few restrictions as to what may be attached at this end, so there is a wide latitude in the groups employed for the 5 various purposes. Generally speaking, as indicated previously primary purposes will be for isolation of the probe reaction product and detection of the probe reaction product. As many probes are able to pass through the cell membrane and react intracellularly, the subject probes can be used to follow the intracellular movement of the targets or determine their situs.

10 [0086] For the purpose of isolation and in some instances identification there will be present an affinity tag or a ligand. Suitable affinity tags or ligands include essentially any tag that can be bound by a cognate ligand or binding partner. Preferred affinity tags/ligands do not substantially interfere with binding of the probe to a target cysteine hydrolase.

15 [0087] Affinity tags are well known to those of skill in the art. Such tags include, but are not limited to biotin with avidin/streptavidin, ligands and their cognate receptors, particularly haptens and antibodies, polyhistidine with Ni-NTA, epitopes and cognate antibodies, and the like.

20 [0088] Certain affinity tags include epitope tags. Epitope tags are well known to those of skill in the art. Moreover, antibodies (intact and single chain) specific to a wide variety of epitope tags are commercially available. These include but are not limited to antibodies against the DYKDDDDK (SEQ ID NO:1) epitope, c-myc antibodies (available from Sigma, St. Louis), the HNK-1_carbohydrate epitope, the HA epitope, the HSV epitope, the His₄, His₅, and His₆ epitopes that are recognized by the His epitope specific 25 antibodies (see, e.g., Qiagen), and the like.

25 [0089] In certain preferred embodiments, the ligand is tagged with a hexahistidine (His₆) epitope tag which is bound by a Cu, Ni, or Co complex. One particularly preferred complex for binding His₆ tags is Ni-NTA (Ni- nitrilotriacetic acid). In particularly preferred embodiments, the affinity tag is a biotin which can then be captured by avidin, 30 streptavidin, or variants thereof.

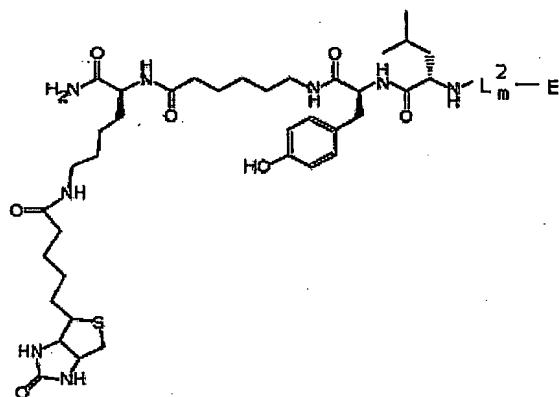
[0090] In certain embodiments, *e.g.*, for the purposes of detection and in some cases isolation, the compounds of this invention (probes) bear a detectable label. Virtually any detectable label can be used as long as it doesn't substantially interfere with the binding of the probe to its target cysteine hydrolase. Larger labels can be accommodated by the use of various linkers/spacers. Thus, other detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Such labels include, but are not limited to, fluorescent dyes (*e.g.*, fluorescein, texas red, rhodamine), fluorescent proteins (green fluorescent protein (GFP), red fluorescent protein (RFP), and the like, *see, e.g.*, Molecular Probes, Eugene, Oregon, USA), radiolabels (*e.g.*, ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold (*e.g.*, gold particles in the 40 -80 nm diameter size range scatter green light with high efficiency), and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

[0091] It will be recognized that fluorescent labels are not to be limited to single species organic molecules, but include inorganic molecules, multi-molecular mixtures of organic and/or inorganic molecules, crystals, heteropolymers, and the like. Thus, for example, CdSe-CdS core-shell nanocrystals enclosed in a silica shell can be easily derivatized for coupling to the molecules of this invention (Bruchez *et al.* (1998) *Science*, 281: 2013-2016). Similarly, highly fluorescent quantum dots (zinc sulfide-capped cadmium selenide) have been covalently coupled to biomolecules for use in ultrasensitive biological detection (Warren and Nie (1998) *Science*, 281: 2016-2018). Quantum dot fluorescent labels are commercially available from Quantum Dot Corporation, Hayward, CA.

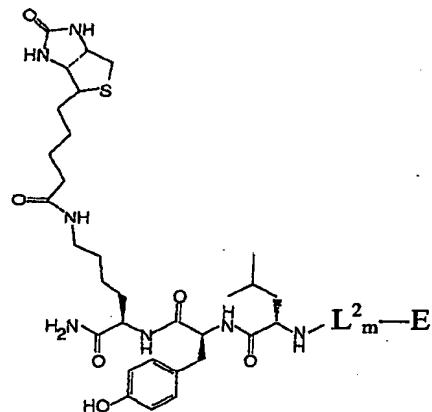
[0092] While in certain embodiments, "A" in formula I, above is a detectable label, other positions can also be labeled in the probe whether "A" is a label or another moiety. Thus, for example, in certain preferred embodiments, the probes are also labeled with a detectable label in addition to the ligand/affinity tag/label and thus provide dual-functionality probes. While, in such cases, the "second" detectable label is preferably a radioactive label (*e.g.* ^3H , ^{125}I , ^{35}S , ^{14}C , ^{32}P , *etc.*), it need not be so limited. The "second label" and the "other" labeling position, is chosen so as not interfere with the binding of

the compound (probe) to a target cysteine protease. Labels (e.g., radioactive labels such as ^3H , ^{125}I , ^{35}S , ^{14}C , ^{32}P , etc.), can be attached in accordance with conventional means, for example, using tyrosine for labeling with a radioactive iodine (^{125}I).

[0093] Illustrative of the compounds of this invention employing various labels, 5 having a an oligopeptide linker and using glutamic acid to link the label, are the following formulae, where E and L^2 have been defined previously and m is 0 when L^2 is a bond and is otherwise 1. Examples, of such embodiments, are illustrated by the probes of formula IV and V.



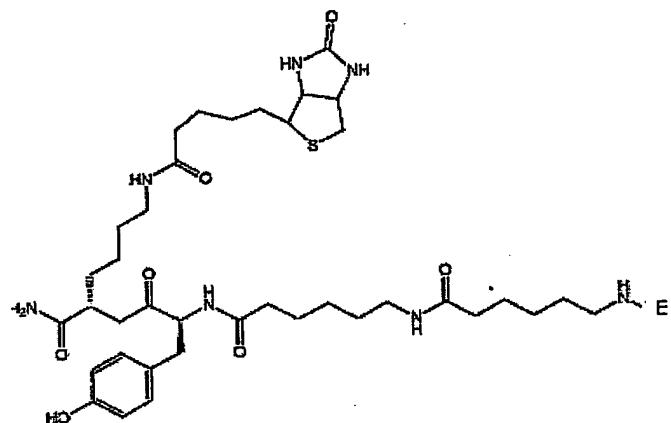
IV



V

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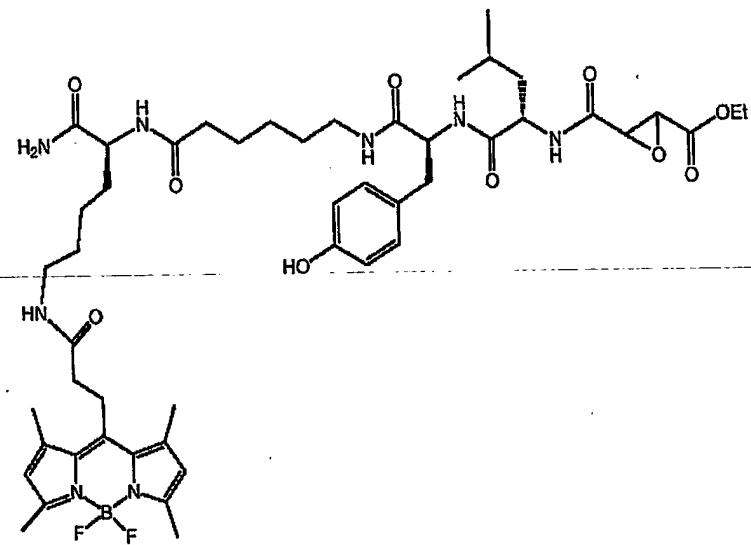
[0094] The following formula VI illustrates L^2 as a linker:



VI

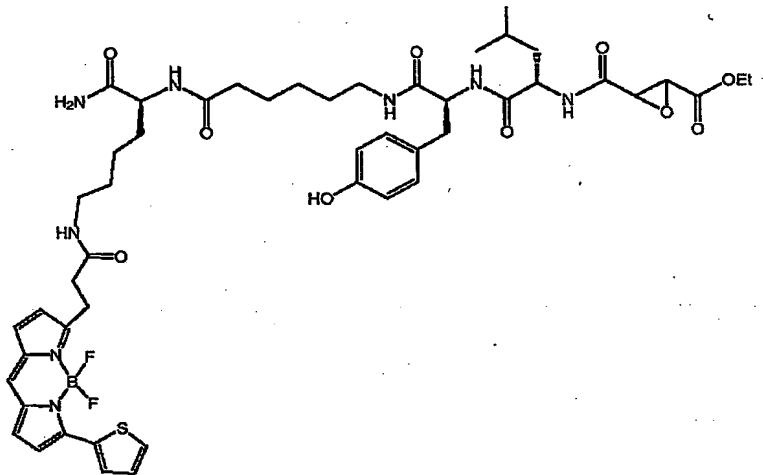
[0095] Particularly preferred probes of this invention are illustrated in Figure 10.

[0096] The following formulae illustrate probes of this invention having an oligopeptide chain linked to a fluorescent moiety. These probes are illustrated by 5 Formulas VII through X (designated BODIPY558/568-DCG-04, BODIPY493/503-DCG-04, BODIPY530/550-DCG-04, and BODIPY588/616-DCG-04, respectively).



VII

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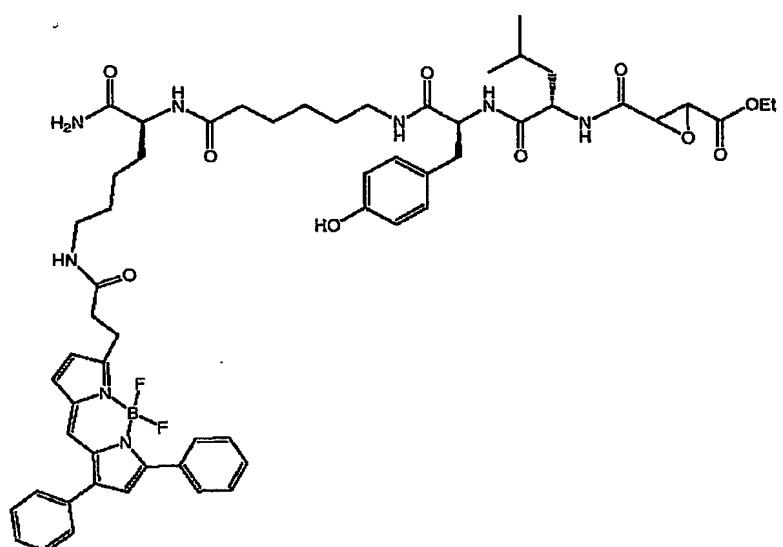
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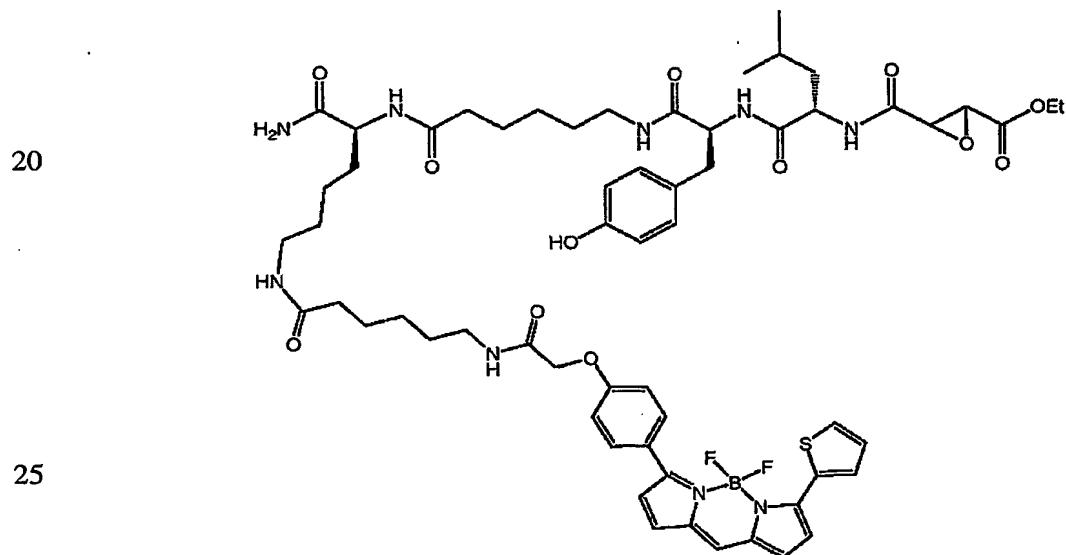
VIII

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IX

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X

II. Probe synthesis.

[0097] Depending on the composition of the probe, various protocols may be used for synthesizing the probe. For the most part, the synthesis will involve the use of synthons or building blocks, particularly where the probe is an oligomer.

5 For preparing oligomers, it will generally be useful to use a solid support and build the oligomer on the solid support in accordance with known methods. Where the probe has only three elements, the reactive group or electrophile, the hydrophobic group or binding specificity group, and the ligand, the synthesis may be performed in solution, where the order of combining the individual components may be varied.

10 [0098] The probes of this invention can be synthesized according to standard methods known to those of skill in the art. It is noted that methods of coupling electrophiles, and other molecules, to peptides are well known. However, in preferred embodiments, particularly where the electrophile is an epoxide, this invention provides an improved synthesis method. Briefly, this method involves a combination of solution and 15 solid phase chemistries. The solution phase synthesis of the epoxide acid building block starting from commercially available diethyl tartrate is shown in Figure 2A. Standard solid-phase peptide chemistry is used to build the peptide portion of the probe (e.g. DCG-04) and related compounds (see, e.g., Figure 2B). This methodology provides a flexible system with which to incorporate virtually any peptide sequence prior to attachment of the 20 electrophilic epoxide. It was a surprising discovery of this invention that the epoxy acid building block was stable to standard solid-phase peptide synthesis cleavage conditions (95% TFA).

25 [0099] The use of solid-phase chemistry also facilitates the synthesis of a diverse library in which, for example, the P2 leucine of DCG-04 is replaced with each of the natural amino acids (except cysteine due to reactivity with the epoxide and methionine due to oxidation).

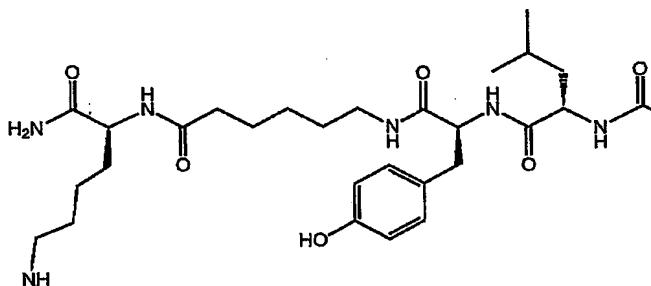
[0100] By having the electrophile with a functionality, either amino or carboxy that can be joined to a solid support and a second functionality for linking to an amino acid, the subject compounds can be synthesized on a solid support by stepwise addition. 30 In the subject compounds the electrophile is linked to the hydrophobic group by an amide bind with the electrophile supplying the carboxyl and the hydrophobic group providing the amino group. The amide group could be in the reverse direction. To some degree, the

order of addition is arbitrary, except that where there are many functionalities on the units, one must devise a protocol for protection and deprotection that restricts bond formation to form the desired linkage. For the purposes of this invention, the direction of the linking functional groups may be in either direction where there is asymmetry, as with amides and 5 esters.

[0101] Fluorophores are readily provided in place of the affinity tag, *e.g.* by synthesizing a free amine version of the probe (*e.g.* Formula XI) and reacting it with the succinamide ester of the fluorophore. Such derivatized fluorophores are readily obtained from Molecular Probes (Oregon, CA).

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15



XI

III. Probe Libraries.

20 [0102] In another embodiment, this invention provides libraries of probes of this invention. Preferred libraries include, at least two, preferably at least five, more preferably at least ten, and most preferably at least twenty-nine different probes as described herein (*e.g.* of formula I-X). In certain preferred probe libraries, each species of probe comprises a different and distinguishable label. Certain preferred probe libraries comprise 25 a library of probes comprising amino acid or dipeptide protease binding/recognition domains. Certain preferred libraries comprise a dipeptide of the form: -Tyr-X- where X is essentially any other amino acid. In one particularly preferred library X includes, essentially any other naturally occurring amino acid and norleucine.

30 [0103] The libraries can be provided in any of a wide variety of formats. Thus, for example, all the members of a probe library can be combined into a single probe mixture. Alternatively, each probe can be provided in a separate container.

[0104] In embodiments, well suited for high throughput screening applications, the probe library is provided as one or more microtiter plates (e.g. 96 well plate, 384 well plate, etc.), with each library member (or several library members) in each well.

Microtiter plate formats are well suited to handling and manipulation using laboratory robotic systems.

[0105] The probes of this invention can be also be provided attached to a substrate. Suitable substrates included, but are not limited to, solid surfaces, membranes, or gels. Substrate materials include, but are not limited to plastics, glass, quartz, metals, ceramics, and the like. In preferred embodiments, the probes (e.g. a probe library) is attached to a single contiguous surface or to a multiplicity of surfaces juxtaposed to each other (e.g. to a collection of beads or other particles).

[0106] When the probe library is attached to a surface it forms a probe array suitable for a wide variety of assays including, but not limited to, fingerprinting tissue cysteine protease activities, providing an activity profile of a cysteine hydrolase, and the like.

[0107] The probes of this invention can be coupled to a substrate according to any of a number of methods well known to those of skill in the art. Such methods include, but are not limited to, simple adsorption cross-linking with the use of linkers, or attachment by way of the affinity tag. In particularly preferred embodiments, the probes are attached to the surface by the affinity tag. Thus, for example, a surface bearing a streptavidin, or a modified streptavidin will bind a biotin affinity tag on the probe. Similarly, a surface bearing a Ni-NTA moiety will bind to a His₆ affinity tag. The selection of the affinity tag and the binding moiety will determine whether or not it is possible to subsequently release the probe(s) from the surface. Thus, for example a Ni-NTA-His₆ coupling is reversible. Similarly, while a biotin-streptavidin coupling is typically not cleavable, monomeric avidin has a reduced binding affinity biotin the bound probe can competitively eluted with high concentrations of biotin (e.g., 2 mM).

[0108] In certain embodiments, the probes of this invention are provided attached to beads and/or a polymeric resin that can be packed into a column. A sample (e.g. crude cell extract) can be run through the column where permitting probe targets to be bound. The bound target can then, optionally, be eluted.

IV. Assays using cysteine hydrolase probes.

[0109] The cysteine hydrolase probes of this invention are useful in a wide variety of contexts. The probes may be specific for a range of different groupings of cysteine hydrolases, across one group of cysteine hydrolases, e.g. CA, CB and CD, or for one or a

5 few cysteine hydrolases. Preferred uses include include, but are not limited to the analysis of cysteine hydrolase activities in crude cell extracts, identification of novel hydrolases, profiling of cysteine hydrolase activity in disease states, screening of candidate compounds for cysteine hydrolase activity, and tracking of cysteine hydrolases in biological fluids and tissue samples.

10 [0110] The probes of this invention can comprise ligands that include an affinity tag (e.g. biotin), ligands that facilitates detection of probe bound target molecules using, e.g., an affinity blot protocol (e.g. Western Blotting and labeling with a ligand specific to the affinity tag). In certain embodiments, a detectable label (e.g. a fluorescent label) is used instead of the affinity tag allowing rapid probe detection using, e.g., various 15 fluorometric methods. The affinity tag or ligand also facilitates the temporary or permanent attachment of the probe(s) to a substrate whereby the probes(s) form effective affinity "chromatography" ligands facilitating isolation and characterization of the bound cysteine hydrolase(s).

20 [0111] For convenience, the ligand- or affinity tag-bearing probes of this invention can also be labeled with a detectable label (e.g. a radioactive label) thereby providing a bi-functional probe. The detectable label facilitates rapid detection of bound target molecules even in crude protein mixtures. For example, analysis of the labeling of DC2.4 lysates (see Example 1) by both affinity blot and label-detection (e.g. auto-radiography) 25 techniques resulted in similar profiles of modified target molecules, highlighting the utility of both techniques. The presence of the affinity tag facilitated rapid isolation and further characterization of the tagged target molecule(s). Ultimately, the ability to use both autoradiography as well as blot techniques enhances the flexibility of the probes of this invention.

30 [0112] In various embodiments, as described below, the libraries of the probes of this invention can be used to identify a cysteine hydrolase and/or to provide a profile of a cysteine hydrolase's specificities (i.e. a hydrolase/protease fingerprint). The activity of individual cysteine hydrolases or a plurality of hydrolases can be profiled in a particular

tissue or collection of tissues to characterize tissue-specific differences in cysteine hydrolase activities, to characterize developmental changes in cysteine hydrolase activities, to characterize changes in cysteine hydrolase activity in response to altered environmental conditions, to characterize changes in cysteine hydrolase activity associated with disease progression, to provide a fingerprint that is a measure of disease stage or progression, and the like.

A) Identifying and/or isolating and/or characterizing cysteine hydrolases.

1) Protease fingerprinting.

[0113] In one embodiment, this invention provides methods of identifying an "activity profile" for a particular cysteine hydrolase or a group of cysteine hydrolases. IN preferred embodiments, such an activity profile comprises a measure of the activity of a plurality of probes of this invention for one or more cysteine hydrolases. Thus, in preferred embodiments, "fingerprinting" generally involves determining the binding of one or more probes of this invention, preferably of a library of probes of this invention to a particular cysteine hydrolase or group of cysteine hydrolases. This can be done using a "direct labeling" assay, however, preferably, a competitive assay is used (e.g. using a known inhibitor of the target protease). This assay generates an activity profile showing the relative binding of each probe comprising the library to the target protease.

[0114] The generation of such a profile for cathepsin H is illustrated herein in Example 1 (Figure 6B). Pre-incubation of purified cathepsin H with the library of compounds followed by ¹²⁵I-DCG-04 labeling resulted in a specificity profile that was remarkably similar to the profile observed for cathepsin B in crude extracts (Figure 6C). The data showed that although the two proteases are quite different in their biological functions, they have similar inhibitor specificity in the S2 pocket.

[0115] Since it is unlikely that two distinct proteases will exhibit identical reactivity across a diverse set of inhibitors, it is possible to use this information from positional scanning inhibitor libraries to generate "specificity fingerprints" for a series of well characterized hydrolases.

[0116] It is believed that creation of a database of cysteine hydrolase inhibitor profiles can be used to establish target identification by labeling of crude protein mixtures

in the presence of compound libraries. The labeling pattern (fingerprint) is read and compared to the database. Identification of similar or the same fingerprint(s) in the database provides an indication of the cysteine hydrolase(s) present in the sample.

2) Identification and characterization of unknown hydrolases.

5 [0117] The methods of this invention can also be used to characterize known cysteine hydrolases and/or to identify, isolate, and/or characterize unknown cysteine hydrolases. These methods involve contacting a biological sample with one or more probes of this invention and detecting binding of the probe(s) with component(s) of the sample. Because the probes of this invention are generally specific to cysteine hydrolases, 10 binding of the probes to a component in the sample initially indicates the presence of a cysteine hydrolase. Use of a library of probes of this invention increases the likelihood of detecting cysteine hydrolases, but requires no assumptions regarding which cysteine hydrolases are present in the sample.

15 [0118] Once specific binding is identified, the affinity tag on the probe(s) can readily be used to capture and isolate the bound cysteine hydrolase. The isolated hydrolase is then readily subjected to further analysis (e.g. tryptic digests, amino acid analysis, mass spectrometry, etc.). The isolation and subsequent analysis of isolated peptides is illustrated in Example 1.

20 [0119] In certain embodiments, the affinity tag is attached to the specificity determinant using a cleavable linker. Cleavable linkers circumvent problems of background proteins and endogenously biotinylated proteins or peptides that non-specifically stick to affinity resins during purification of protease targets from crude protein extracts. The cleavable linker is used to join the probe to a resin (e.g. Affigel from BioRad) to create an affinity resin. The "affinity resin" can be directly incubated with 25 crude protein extracts and then be stringently washed (e.g., high SDS, low pH, boiling, etc.) to assure elimination of non-specific background proteins or peptides. This is followed by release of specific peptide products by trypsin digestion, acid hydrolysis, mild oxidation, or photorelease resulting in cleavage of the linker and release of the probe modified peptides. This method coupled with trypsin digestion and washing of the affinity 30 resin prior to specific elution allows direct mass spectrometry of only active site peptides of modified cysteine proteases targets. The methods therefore can be used to obtain

sequence information for multiple active site peptides simultaneously without the need for resolution by gel electrophoresis.

B) Profiling protease activity in a tissue (activity monitoring).

[0120] One or more probes of this invention can be used to provide a cysteine hydrolase "activity profile" in one or more tissue types. Essentially any cell, tissue, or biological fluid can be subjected to such profiling as long as it contains one or more cysteine hydrolases. Selection of particular cells or tissues for such profiling allows cysteine hydrolase expression to be evaluated in a wide variety of contexts as indicated above. A few examples are illustrated below and in Example 1.

1) Profiling disease progression.

[0121] One or a plurality of probes of this invention can be used to profile the progression of a disease. The subject probes preferably find application where a disease state results in the up or down regulation of the expression or activity of at least one of the enzymes to which the probes bind. While essentially any disease state can be profiled, disease progressions that are expected to involve cysteine hydrolase activity are particularly well suited for such profiling. One class of such diseases includes diseases characterized by tissue remodeling (e.g. various cancers, in particular invasive and/or metastatic cancers, rheumatoid arthritis, osteoporosis, and the like).

[0122] The use of the probes of this invention to profile progression of a cancer is illustrated in Example 1. While this Example uses a single, broadly reactive probe, the same methodologies can be used with a library of probes of this invention to provide a more detailed profile of the disease progression. Where the probes have different specificity the pattern of binding of each of the probes can be used to identify the individual enzymes or classes of enzymes and the level of activity for each of the enzymes or classes of enzymes.

[0123] As described in Example 1, the mouse skin model of multi-stage carcinogenesis was profiled using a single probe of this invention (DCG-04). Ten cell lines representing various steps in the progression from benign skin cell (C5N) to highly invasive spindle cell carcinomas (CarB and CarC) were used to analyze global changes in activity of cathepsins throughout this multi-stage carcinogenesis model. The carcinoma

progression also included benign papilloma cell lines P6, PDV and PDV-C57, and more invasive squamous cell carcinoma cell lines B9, A5, D3. Equal amounts of protein from each cell lysate were labeled with both the broadly reactive probe, ¹²⁵I-DCG-04, as well as the cathepsin B-specific probe, ¹²⁵I-MB-074 at pH 5.5 (Figure 5). The results showed that 5 several protease activities, including cathepsin B, dramatically fluctuate across the panel of cell lines.

[0124] The data provided in Example 1 illustrate that cells isolated from different tumor sources have different protease activity profiles. These profiles can be used, *e.g.*, in a database to relate the profile to various aspects of the tumor cells, for example, the 10 aggressiveness of the disease, the response to treatment, changes in tumor status, etc. Without being bound to a particular theory, it is believed that this signature of protease activity may in fact be unique to each cell and/or tumor much the same way genomics studies have shown that individual tumor cells have unique global gene expression profiles.

15 [0125] These methods can be used to profile the progression of essentially any disease state in which cysteine hydrolases play a role and their regulation and activity vary with the status of the disease, particularly as it relates to therapeutic treatment, advances and regressions or remissions. One may use an established model system or create an independent model system. Tissue biopsies, cells, and the like can be obtained, for 20 example, from patients diagnosed at particular stages of a particular disease and characteristic profiles can be determined for the various disease stages.

[0126] Cysteine hydrolase expression/activity produced using particular probes of this invention in tissues obtained from characteristic stages of a disease progression can be entered into a database of such profiles. This database, or particular entries in such a 25 database, can provide a reference or characteristic profile useful for staging or diagnosing or evaluating the prognosis of a disease. In such an embodiment, a sample is obtained from a subject and a cysteine hydrolase activity profile is determined using one or more particular probes of this invention. The resulting activity profile is then compared to a one or more "reference" profiles, *e.g.* stored in a database. If the measured profile is 30 sufficiently similar to, or "identical" with, a reference profile and that reference profile is characteristic of a particular disease, particular disease stage, or prognosis, it can be inferred that the subject exhibits that disease, disease stage, or prognosis. Such a

determination, need not be definitive of such a disease, disease stage, or prognosis, but can simply serve as a component of a differential diagnosis which can utilize known disease indicators. The determination of disease state or prognosis can then inform decisions regarding a treatment regimen (e.g. the decision whether or not to use chemotherapy and/or radiotherapy in addition to surgery in the treatment of a cancer patient, etc.).

5 2) Profiling across tissue types.

[0127] One or more probes of this invention can be used to profile cysteine hydrolase expression in a variety of tissue types. Thus, hydrolase activity in diseased tissues can be compared to healthy tissues, hydrolase activity in differentiated cells can be 10 compared to undifferentiated cells, changes in cysteine hydrolase activity in response to environmental conditions or drugs, and the like can be assayed. Such profiles can be determined using a single probe, however in most cases, a probe library is used.

[0128] The creation of such a tissue profile using a probe library is illustrated in Example 1. In this Example, a small library of compounds is employed in which the 15 peptide recognition portion of the molecule was varied. A complete scanning library consisting of 18 natural amino acids and the isosteric methionine analog norleucine was constructed substituting the various amino acids for leucine in DCG-04. This library of inhibitors was used to create profiles of inhibitor specificity for proteases targeted by DCG-04 and MB-074 (Figure 6).

20 [0129] Competition analysis was used to determine the potency of each member of the P2 scanning library towards multiple protease targets. Lysates from DC2.4 cells were pre-incubated with 50 μ M of each of the 19 DCG library members and residual activity measured for multiple proteases using 125 I-DCG-04 (Figure 6A). In general, residues containing non-charged aliphatic side chains, isoleucine (I), leucine (L; DCG-04) and 25 norleucine (n), showed highest activity and the lowest amount of specificity across the profile of polypeptides. More interesting was the apparent selectivity of several DCG family compounds for a subset of labeled polypeptides. For example, the valine containing compound competed for polypeptides 1, 2 and cathepsin B but had little effect on the remaining compounds. In contrast, both the phenylalanine and tyrosine containing 30 compounds showed specificity for polypeptides 2, 3, 4, and 5. Furthermore, while the aspartic acid and glycine containing compounds showed relatively poor activity overall,

they showed some degree of specificity against polypeptide 2. Similar methods can be used to profile essentially any tissue.

C) Screening for modulators of cysteine hydrolase activity.

[0130] The methods described herein for profiling tissue types or for profiling 5 individual hydrolases can also be used to screen for modulators of cysteine hydrolase activity. Basically, the biological sample is contacted with one or more test agents. The sample is then profiled for cysteine hydrolase activity with one or more probes of this invention as described above. In addition, different concentrations of modulators can be used to establish the dose response of modulation of cysteine protease activity. This 10 method can also be used to determine the selectivity of a given modulator with respect to all cysteine protease targets of one or more probes of this invention.

[0131] The sample (*e.g.* crude cell extract) can be contacted with the agent directly. Alternatively, where the sample is a cell line, the cell line can be contacted with the test agent, or cultured in the presence of the test agent. In other embodiments, the test 15 agent can be administered to an animal and biological samples derived from the animal are profiled as described above.

[0132] When the sample contacted with the test agent shows a cysteine hydrolase activity profile different from the profile obtained from a negative control (*e.g.* a sample contacted with a lower amount of test agent or no test agent) it is inferred that the test 20 agent modulates cysteine hydrolase activity. The assays of this invention are typically scored as positive where there is a difference between the activity seen with the test agent present and the (usually negative) control, preferably where the difference is statistically significant (*e.g.* at greater than 80%, preferably greater than about 90%, more preferably greater than about 98%, and most preferably greater than about 99% confidence level). 25 Most preferred "positive" assays show at least a 1.2 fold, preferably at least a 1.5 fold, more preferably at least a 2 fold, and most preferably at least a 4 fold or even a 10-fold difference from the negative control.

1) Agents for screening: Combinatorial libraries (e.g., small organic molecules)

[0133] Virtually any agent can be screened according to the methods of this invention. The term "test agent" refers to any agent that is to be screened for a desired 5 activity. The "test composition" can be any molecule or mixture of molecules, optionally in a suitable carrier. Such agents include, but are not limited to nucleic acids, proteins, sugars, polysaccharides, glycoproteins, lipids, and small organic molecules, both naturally occurring and synthetic. Preferred test agents include small organic molecules.

[0134] Conventionally, new chemical entities with useful properties are generated 10 by identifying a chemical compound (called a "lead compound") with some desirable property or activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. The current trend is to shorten the time scale for all aspects of drug discovery. Because of the ability to test large numbers quickly and efficiently, high throughput screening (HTS) methods are replacing conventional lead 15 compound identification methods.

[0135] In one preferred embodiment, high throughput screening methods involve providing a library containing a large number of potential therapeutic compounds 20 (candidate compounds). Such "combinatorial chemical libraries" are then screened in one or more assays, as described herein to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity (e.g. ability to modulate a cysteine protease activity, or activity profile). The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

[0136] A combinatorial chemical library is a collection of diverse chemical 25 compounds generated by either chemical synthesis or biological synthesis by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide (e.g., murein) library is formed by combining a set of amino acids in multiple different orders for a given number of amino acid units. Millions of chemical compounds can be synthesized through such 30 combinatorial mixing of chemical building blocks. For example, one commentator has observed that the systematic, combinatorial mixing of 100 interchangeable chemical

building blocks results in the theoretical synthesis of 100 million tetrameric compounds or 10 billion pentameric compounds (Gallop *et al.* (1994) 37(9): 1233-1250).

[0137] Preparation of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to,

5 peptide libraries (*see, e.g.*, U.S. Patent 5,010,175, Furka (1991) *Int. J. Pept. Prot. Res.*, 37: 487-493, Houghton *et al.* (1991) *Nature*, 354: 84-88). Peptide synthesis is by no means the only approach envisioned and intended for use with the present invention. Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (PCT Publication No WO 91/19735, 26 Dec.

10 1991), encoded peptides (PCT Publication WO 93/20242, 14 Oct. 1993), random bio-oligomers (PCT Publication WO 92/00091, 9 Jan. 1992), benzodiazepines (U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, (1993) *Proc. Nat. Acad. Sci. USA* 90: 6909-6913), vinylogous polypeptides (Hagihara *et al.* (1992) *J. Amer. Chem. Soc.* 114: 6568), nonpeptidal peptidomimetics with a Beta-D-

15 Glucose scaffolding (Hirschmann *et al.*, (1992) *J. Amer. Chem. Soc.* 114: 9217-9218), analogous organic syntheses of small compound libraries (Chen *et al.* (1994) *J. Amer. Chem. Soc.* 116: 2661), oligocarbamates (Cho, *et al.*, (1993) *Science* 261:1303), and/or peptidyl phosphonates (Campbell *et al.*, (1994) *J. Org. Chem.* 59: 658). *See, generally*, Gordon *et al.*, (1994) *J. Med. Chem.* 37:1385, nucleic acid libraries (*see, e.g.*, Strategene, Corp.), peptide nucleic acid libraries (*see, e.g.*, U.S. Patent 5,539,083) antibody libraries (*see, e.g.*, Vaughn *et al.* (1996) *Nature Biotechnology*, 14(3): 309-314), and PCT/US96/10287), carbohydrate libraries (*see, e.g.*, Liang *et al.* (1996) *Science*, 274: 1520-1522, and U.S. Patent 5,593,853), and small organic molecule libraries (*see, e.g.*, benzodiazepines, Baum (1993) C&EN, Jan 18, page 33, isoprenoids U.S. Patent

20 25 5,569,588, thiazolidinones and metathiazanones U.S. Patent 5,549,974, pyrrolidines U.S. Patents 5,525,735 and 5,519,134, morpholino compounds U.S. Patent 5,506,337, benzodiazepines 5,288,514, and the like).

[0138] Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.*, 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY,

30 Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA).

[0139] A number of well known robotic systems have also been developed for solution phase chemistries. These systems include, but are not limited to, automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Hewlett-Packard, Palo Alto, Calif.) which mimic the manual synthetic operations performed by a chemist and the VentureTM platform, an ultra-high-throughput synthesizer that can run between 576 and 9,600 simultaneous reactions from start to finish (*see* Advanced ChemTech, Inc. Louisville, KY)). Any of the above devices are suitable for use with the present invention. The 10 nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art. In addition, numerous combinatorial libraries are themselves commercially available (*see*, e.g., ComGenex, Princeton, N.J., Asinex, Moscow, RU, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, 15 Columbia, MD, *etc.*).

2) High Throughput Screening.

[0140] Any of the assays for compounds modulating the activity of cysteine hydrolases described herein are amenable to high throughput screening. The biological samples utilized in the methods of this invention need not be contacted with a single test 20 agent at a time. To the contrary, to facilitate high-throughput screening, a single sample may be contacted by at least two, preferably by at least 5, more preferably by at least 10, and most preferably by at least 20 test compounds. If the sample scores positive, it can be deconvoluted, *e.g.*, subsequently tested with a subset of the test agents until the agents having the activity are identified.

25 [0141] Robotic high throughput screening systems are commercially available (*see*, *e.g.*, Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, *etc.*). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in 30 detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization. The

manufacturers of such systems provide detailed protocols the various high throughput. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding, and the like.

D) Assay formats.

5 [0142] Preferred probes of this invention can form an essentially irreversible bond with their target cysteine hydrolases. Because the target molecule/probe complex is so stable, it can be subjected to a wide variety of chemical procedures including, but not limited to, a wide variety of methods used for protein purification and analysis (*e.g.*, gel electrophoresis, anion exchange chromatography reverse phase high performance liquid chromatography (HPLC), capillary electrophoresis, entropic trap electrophoresis, *etc.*). In particularly preferred embodiments, the labeled probe/target complex is analyzed using gel electrophoresis and/or Western blotting methods, *e.g.* as described in Example 1.

10 [0143] In certain embodiments, the assays of this invention involve direct labeling of the target cysteine hydrolase (*e.g.* with a radiolabeled probe of this invention) or indirect labeling, (*e.g.* a competition assay). In a direct labeling assay, the labeled probe is contacted with the biological sample under conditions where the probe can specifically bind to its target cysteine hydrolase(s) if they are present. Typically, the labeled cysteine hydrolase(s) will be separated, *e.g.* using SDS-PAGE, 2-D electrophoresis, *etc.*, and the label is detected (*e.g.* using autoradiography for a radioactive label) to provide an indication of the presence and/or amount of labeled target.

15 [0144] In an indirect assay, such as competitive assay, the probe(s) of this invention are contacted with the biological sample during or after contacting of the sample with a reagent that specifically binds to that sample. The reagent can be a probe of this invention or a different type of probe. Probe binding to the target is a function of the relative affinity of the probe to the target as compared to the competing reagent. Either the probe or the competing reagent can be labeled and assayed. A detailed protocol for a competitive binding assay is provided in Example 1.

20 [0145] In certain embodiments, the probe(s) of this invention are immobilized on a solid support. The sample is labeled (*e.g.* with a radioactive label) and contacted to the probes which then act as an affinity matrix specifically binding the target cysteine

hydrolase(s). Detection of the bound labeled sample components provides an indication of binding.

[0146] In such an embodiment, the probe rather than the sample can be labeled. By employing cleavable linkers and releasing the probes from the solid support, the sample components that are not bound by the probe will show different mobility in an electrophoretic gel and are easily distinguished from the target-bound probes. Solid-phase assays of this sort are particularly well suited for high-throughput screening systems. It is also contemplated that such solid-phase assays can be scaled down to "chip-based" formats for rapid screening. Various "lab on a chip" formats are well known to those of skill in the art (see, e.g., U.S. Patents 6,132,685, 6,123,798, 6,107,044, 6,100,541, 6,090,251, 6,086,825, 6,086,740, 6,074,725, 6,071,478, 6,068,752, 6,048,498, 6,046,056, 6,042,710, and 6,042,709) and may readily be adapted to the assays of this invention.

[0147] Assays of this invention are also amenable to solution phase chemistries. In one such embodiment, the biological sample and the probe(s) of this invention are labeled with different detectable labels. When the probe(s) bind a target, the target and probe labels co-localize. Detection of the co-localized labels provides a measurement of bound cysteine hydrolase. The co-localized labeled entities can be isolated and captured using the affinity tag on the probe and then subjected to subsequent analysis.

F) Assay Controls.

[0148] The assays of this invention preferably include a control for non-specific binding. One particularly preferred control comprises a biological sample in which the proteins are denatured (e.g. by heating). Apparent signals generated in such a control are discounted (e.g. subtracted) from the signals read in the test assay. After such a "subtraction" the remaining signal is presumably due to specific binding of the probe(s) to their target proteins.

G) Preferred biological samples.

[0149] The biological samples, used herein, include, but are not limited to samples obtained from an organism, from components (e.g., cells or tissues) of an organism, and/or from *in vitro* cell or tissue cultures. The sample can be of any biological tissue or fluid (e.g. blood, serum, lymph, cerebrospinal fluid, urine, sputum, etc.). Biological samples

can also include organs or sections of tissues such as frozen sections taken for histological purposes. In certain embodiments, the biological samples include crude cell extracts. The extracts can include essentially unpurified cell lysates. The cell lysates can be treated (e.g. centrifuged) to remove particulate matter. Alternatively, the crude cell extracts can 5 comprise isolated cellular "total" protein.

[0150] The biological samples can be derived from any organism that comprises a cysteine hydrolase. Such organisms include, but are not limited to various prokaryotes and essentially all eukaryotic organisms. Preferred organisms include bacteria, fungi, plants, invertebrates and vertebrates. Particularly preferred organisms include mammals 10 (e.g. a rodent, largomorph, murine, bovine, canine, equine, non-human primate, human, etc.).

V. Databases of cysteine protease activity profiles.

[0151] In certain embodiments, the methods of this invention further comprise listing the identified cysteine hydrolases and their activity profiles (as determined by a 15 particular set of probes) in a database identifying activity profiles for various proteins. Similarly, activity profiles for various tissues can also be entered into databases associating tissues with activity profiles for particular activity probes or sets of activity probes.

[0152] The data structures produced by the methods of this invention, or the 20 members of such data structures (i.e., the activity profiles) can be used as reference objects in database searches. Thus, it is possible to use the database to store, retrieve, search and identify similar or identical activity profiles. Comparison of a profile obtained in an assay with a database of profiles may provide an indication as to the cysteine hydrolase composition of the sample, and/or of the physiological state or healthy of the organism 25 from which the sample is derived.

[0153] The term "database", as used herein, refers to a means for recording and retrieving information. In preferred embodiments the database also provides means for sorting and/or searching the stored information. The database can comprise any 30 convenient media including, but not limited to, paper systems, card systems, mechanical systems, electronic systems, optical systems, magnetic systems or combinations thereof. Preferred databases include electronic (e.g. computer-based) databases. Computer

systems for use in storage and manipulation of databases are well known to those of skill in the art and include, but are not limited to "personal computer systems", mainframe systems, distributed nodes on an inter- or intra-net, data or databases stored in specialized hardware (e.g. in microchips), and the like.

5 **VI. Probe kits.**

[0154] This invention also provides kits for practice of the methods described herein. In certain embodiments the kits comprise a container containing one or more of the probes of this invention. In particularly preferred embodiments the kits comprise a plurality of probes of this invention (e.g. a probe library). In certain embodiments, the 10 probe(s) are provided attached to a solid support. The kits can, optionally, further include one or more known inhibitors (e.g. suicide substrate) of a cysteine hydrolase.

[0155] The kits may optionally include any reagents and/or apparatus to facilitate practice of the methods described herein. Such reagents and apparatus include, but are not limited to buffers, instrumentation, microtiter plates, labeling reagents streptavidin or 15 biotin conjugated substrates, PAGE gels, blotting membranes, reagents for detecting a signal, and the like.

[0156] In addition, the kits can include instructional materials containing directions (i.e., protocols) for the practice of the methods of this invention. Preferred instructional materials provide protocols for utilizing the kit contents for screening for 20 cysteine hydrolase activity and/or for activity fingerprinting as described herein. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical 25 media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXAMPLES

[0157] The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1Epoxide Electrophiles As Activity-Dependent Cysteine Protease Profiling And Discovery Tools

[0158] This example illustrates the design and use of chemical probes that can be used to broadly track activity of cysteine proteases. The structure of the general cysteine protease inhibitor E-64 was used as a scaffold. Analogs were synthesized by varying the core peptide recognition portion while adding affinity tags (biotin and radio-iodine) at distal sites. The resulting probes containing a P2 leucine residue (DCG-03 and DCG-04) targeted the same broad set of cysteine proteases as E-64 and were used to profile these proteases during the progression of a normal skin cell to a carcinoma. A library of DCG-04 derivatives was constructed in which the leucine residue was replaced with all natural amino acids. This library was used to obtain inhibitor activity profiles for multiple protease targets in crude cellular extracts. Finally, the affinity tag of DCG-04 allowed purification of modified proteases and identification by mass spectrometry.

[0159] This example thus illustrates a simple and flexible method for functionally identifying cysteine hydrolases while simultaneously tracking their relative activity levels in crude protein mixtures. The probes described herein were used to determine relative activities of multiple proteases throughout a defined model system for cancer progression. Information obtained from libraries of affinity probes provided a rapid method for obtaining detailed functional information without the need for prior purification/identification of targets.

Results and Discussion:Design and synthesis of DCG-04.

[0160] The natural product E-64 is a promiscuous irreversible cysteine protease inhibitor that is broadly reactive toward the papaine family of cysteine proteases (Barrett and Hanada (1982) *Biochem. J.*, 201: 189-198) (Figure 1). Its leucine sidechain mimics the P2 amino acid of a substrate, occupying the target's S2 binding pocket while the agmatine moiety binds in the S3 position (Matsumoto *et al.* (1999) *Biopolymers* 51: 99-107). Rich *et al.* synthesized JPM-565 (Figure 1), a derivative in which a tyramine moiety replaces the agmatine side chain of E-64 (Meara and Rich (1996) *J. Med. Chem.* 39, 3357-

3366; Shi *et al.* (1992) *J. Biol. Chem.* 267, 7258-7262). This closely related compound was found to have similar class-specific reactivity for cysteine proteases as E-64. Since the P2 position of a substrate is considered to be the main specificity determinant for many cysteine proteases, we reasoned that further extension of the non-prime binding portion of 5 JPM-565 would not significantly perturb binding affinity for a target protease. In addition, modification to the non-prime side binding element of the E-64 derivative CA-074 had little effect on binding to cathepsin B (Bogyo *et al.* (2000) *Chem Biol.*, 7: 27-38; 26. Bogyo *et al.* (1997) *Proc. Natl. Acad. Sci., USA*, 94: 6629-6634). Elaboration of the peptide portion of E-64 allowed both incorporation of an affinity tag as well as attachment of the 10 compound to a solid support. The resulting bi-functional compounds, DCG-03 and DCG-04, contain both the iodinatable phenol ring of JPM-565 and the additional affinity site created by incorporation of a sidechain biotinylated lysine residue (Figure 1). Addition (DCG-04) or removal (DCG-03) of an amino hexanoic acid spacer between the affinity site and the electrophile was used to determine the space requirement for binding and 15 recognition of the affinity label by support-bound avidin.

[0161] Peptide epoxides were synthesized using a combination of solution and solid phase chemistries. The solution phase synthesis of the epoxide acid building block starting from commercially available diethyl tartrate is shown in Figure 2A. Standard solid-phase peptide chemistry was used to build the peptide portion of DCG-04 and related 20 compounds (Figure 2B). This methodology provides a flexible system with which to incorporate virtually any peptide sequence prior to attachment of the electrophilic epoxide. Surprisingly, the epoxy acid building block was stable to standard solid-phase peptide synthesis cleavage conditions (95% TFA). The use of solid-phase chemistry also allowed the synthesis of a diverse library in which the P2 leucine of DCG-04 was replaced with 25 each of the natural amino acids (except cysteine due to reactivity with the epoxide and methionine due to oxidation). The non-natural amino acid norleucine was used as an isosteric methionine analog. The results obtained using this 19 member library of compounds are described below.

DCG-04 is an activity-dependent affinity label.

30 [0162] Dendritic cells express relatively high levels of lysosomal cathepsins, making them a logical source of material for establishing parameters for the use of DCG-

04. Figure 3 shows the labeling profile of polypeptides modified by incubation with either DCG-03, DCG-04, ^{125}I -DCG-03, or ^{125}I -DCG-04 followed by SDS-PAGE analysis.

Radio-iodinated (autoradiogram) and non-radio-iodinated (blot) DCG-03 and DCG-04 labeled multiple polypeptides in the range of 20-40 kDa.

5 [0163] Although the intrinsic reactivity of the epoxide electrophile portion of DCG-04 towards free thiols is quite poor, we wanted to determine if DCG-04 and its derivatives were capable of non-specific alkylation of proteins in crude cellular extracts. A preheating control was used to reveal non-specific labeling, with the assumption that denatured, inactive proteins modified by DCG-03 and DCG-04 represent nonspecific 10 modifications. Enzymatically active proteins were deduced by subtraction (Figure 3). Labeling of all of the major species in the 20-40 kDa size range was lost upon heat denaturation of samples prior to addition of compounds suggesting that labeling is dependent on enzymatic activity and that these bands correspond to the major proteases in the extract. Several higher molecular weight species were observed by affinity blotting of 15 both denaturing controls and samples in which no inhibitor was added. These species are likely to represent non-specific alkylations and endogenously biotinylated proteins.

[0164] Comparison of labeling, at neutral (pH 7.4) and at the acidic pH of the lysosome (pH 5.5), indicated that several of the modified polypeptides in the 30kDa size range required reduced pH for activity. This result is consistent with reported findings that 20 several lysosomal cysteine proteases either reversibly or irreversibly lose activity upon deacidification of lysosomal compartments (Barrett *et al.* (1998) *Handbook of Proteolytic Enzymes*, Academic Press, San Diego).

[0165] Analysis of the labeling of DC2.4 lysates by both affinity blot and auto-radiography techniques resulted in similar profiles of modified polypeptides, highlighting 25 the utility of both techniques. However, the auto-radiogram showed exclusive modification of enzymatically active polypeptides by radiolabeled forms of DCG-03 and DCG-04. Addition of the rather bulky iodine atom to DCG-03 and DCG-04 had only a modest effect on target modification yet resulted in compounds with dramatically reduced background labeling and increased sensitivity. Ultimately, the ability to use both 30 autoradiography as well as blot techniques enhances the flexibility of these protease detection reagents and further highlights the utility of bi-functional inhibitors.

DCG-04 targets cysteine proteases inhibited by E-64 and JPM-565.

[0166] Both direct labeling and indirect competition experiments were performed to confirm that DCG-04 reacts with a similar subset of proteases to the parent compounds E-64 and JPM-565. An indirect competition experiment was required to determine the 5 polypeptides modified by E-64 since it lacks an affinity label. Extracts from the dendritic cell line DC2.4 were preincubated with increasing concentration of E-64 followed by labeling with ¹²⁵I -DCG-04. Final labeling intensity was used to indirectly monitor extent of polypeptide modification by E-64. The competition revealed that all polypeptides labeled by ¹²⁵I-DCG-04 are effectively competed by E-64 indicating that the two 10 compounds target the same subset of proteases (Figure 4A). A similar competition experiment was performed using the cathepsin B specific inhibitor MB-074 (Bogyo *et al.* (2000) *Chem Biol.*, 7: 27-38). These results positively identified the diffuse 30 kDa polypeptide (labeled cat B in Figure 4A) as cathepsin B (data not shown).

[0167] Comparison of the specificity of DCG-03, DCG-04 and JPM-565 was 15 accomplished using direct labeling of DC2.4 cell lysates. Labeling profiles obtained for ¹²⁵I -DCG-03, ¹²⁵I -DCG-04 and ¹²⁵I -JPM-565 were identical for all three probes and indicated that each targeted polypeptides in the 20-40 kDa size range (Figure 4B). Analysis of non-radiolabeled DCG-03, DCG-04 and JPM-565 treated extracts again 20 showed the similarity of the blotting and autoradiography detection systems. As expected, JPM-565, which lacks a biotin label, showed no labeling as detected by affinity blotting. Together these results establish that modifications to the extended binding portion of the 25 E-64 family of compounds have little effect on selectivity or potency. However, this region of the inhibitor may still play an important role in establishing specificity of binding when equipped with the proper recognition sequence. Future work is aimed at exploring the use of extended peptide recognition motifs to fine tune selectivity of the DCG family of inhibitors for specific protease targets.

Profiling Applications:

[0168] The aforementioned methods established the initial parameters for use of the general cysteine protease labels DCG-03 and DCG-04. We next wanted to apply these 30 techniques to profile the activity and specificity of cysteine proteases in several different model systems. The broadly reactive probe DCG-04 was used to generate activity profiles

of multiple protease targets both in a model for disease progression and throughout multiple tissue types. Similarly, activity profiles were generated using the cathepsin B specific probe MB-074 to provide complementary information for a single, well-defined cysteine protease target. This information was also used to positively establish the identity 5 of cathepsin B in the DCG-04 labeling profiles. To obtain more detailed functional information for DCG-04 modified proteases, inhibitor specificity profiles were generated using a library of DCG-04 analogs in total cellular extracts. The same libraries were also used in conjunction with the cathepsin B-specific probe, ¹²⁵I-MB-074, as well as with purified cathepsin H to determined specificity profiles for individual target proteases.

10 These results are described below.

Profiling across disease progression using DCG-04 and MB-074.

[0169] The mouse skin model of multi-stage carcinogenesis has been well-studied genotypically and phenotypically, has discrete steps in the progression, but lacks information on cysteine protease involvement (Kemp *et al.* (1994) *Cold Spring Harbor Symp. Quant. Biol.* 59: 427-434; Yuspa *et al.* (1994) *J. Investigative Dermatol.*, 103: 90S-95S). The role of cathepsins in tumor biology has mostly focused on cathepsin B and L. Up-regulated levels of both cathepsin B and L have been shown to correlate with an invasive phenotype (Yan *et al.* (1998) *Biol. Chem.*, 379: 113-123; Baricos *et al.* (1988) *Biochem. J.* 252: 301-304). Furthermore, cathepsins B and L are secreted by many types 20 of tumorigenic cells and treatment of invasive cells with the cysteine protease inhibitor E-64 results in a block in cellular invasion into a synthetic matrix (Linebaugh *et al.* (1999) *Europ. J. Biochem.*, 264: 100-109; Mason *et al.* (1987) *Biochem. J.* 248: 449-454). These data indicate that cathepsins are likely to play an important role in the metastatic process.

[0170] Ten cell lines representing various steps in the progression from benign 25 skin cell (C5N) to highly invasive spindle cell carcinomas (CarB and CarC) were used to analyze global changes in activity of cathepsins throughout this multi-stage carcinogenesis model. The carcinoma progression also includes benign papilloma cell lines P6, PDV and PDV-C57, and more invasive squamous cell carcinoma cell lines B9, A5, D3. Equal amounts of protein from each cell lysate were labeled with both the broadly reactive 30 probe, ¹²⁵I-DCG-04, as well as the cathepsin B-specific probe, ¹²⁵I-MB-074 at pH 5.5

(Figure 5). The results show that several protease activities, including cathepsin B, dramatically fluctuate across the panel of cell lines.

[0171] The broadly reactive probe ^{125}I -DCG-04 highlights the activity of several proteases in the lysosomal cysteine protease size range in each of the cell types (Figure 5A). The benign cell lines C5N and P6 both contain multiple labeled polypeptides between 28 and 45 kDa, however, the labeling intensity observed for the P6 line is dramatically increased for all polypeptides in this range. Interestingly, the major difference between these cell lines is an activating mutation in the *ras* gene (Quintanilla *et al.* (1991) *Carcinogenesis* 12: 1875-1881). It has been previously shown that various classes of proteases, including the cathepsins, are upregulated downstream of Ras; however, these studies were limited to analysis of expression levels of cathepsin B and H (Kim *et al.* (1998) *International J. Cancer* 79: 324-333).

[0172] The papilloma cell lines PDV, and PDV-C57 show nearly identical patterns of labeling (Figure 5A). However, these profiles are dramatically different than the profile observed for C5N and P6 lysates. A predominant 30 kDa polypeptide (cathepsin B; see below) is modified along with a less intensely labeled 21 kDa polypeptide. The squamous cell carcinoma cell lines B9, A5 and D3 result in a similar profile of polypeptides modified. While all three lines are nearly identical cancer cells types, only B9 shows appreciable labeling of the major 30 kDa and 21 kDa polypeptides. Similarly, the two highly invasive spindle cell carcinomas Car B and Car C show similar, but not identical, labeling profiles. The 21 kDa species, in particular, shows differential labeling in the two cell types. These findings illustrate that cells isolated from different tumor sources have different protease activities. This signature of protease activity may in fact be unique to each cell and/or tumor much the same way genomics studies by Browne and colleagues have shown that individual tumor cells have unique global gene expression profiles (Alizadeh and Staudt, (2000) [see comments]. *Nature* 403: 503-511).

[0173] The cathepsin B-specific label ^{125}I -MB-074 was used to directly examine the profile of cathepsin B activity in the same collection of cells described above (Figure 5B). This probe has been found to label cathepsin B in a highly specific manner (Bogyo *et al.* (2000) *Chem Biol.*, 7: 27-38). Labeling of cathepsin B dramatically changed across the profile of cell types with the greatest activity observed for the PDV and PDV-C57 lines. Furthermore, the apparent molecular weight as well as the sharpness of the cathepsin B

band differed for the benign and spindle cell carcinomas suggesting that this enzyme is modified differently in these cell types. This change in migration for cathepsin B may be due to changes in glycosylation or other post translational modifications. Cathepsin B has been found to exist as different isoforms of differing pIs in various tumor cells as a result of changes in glycosylation and trafficking (Moin *et al.* (1998) *Biol. Chem.*, 379: 1093-1099). Changes in the post-translational modification of cathepsin B is likely to effect the localization of active forms of the enzyme and therefore may play an important role in the control of cathepsin B activity in tumors (Moin *et al.* (1998) *Biol. Chem.*, 379: 1093-1099). Overall, the results obtained from labeling with ¹²⁵I-MB-074 further highlight the variability of cathepsin B activity found in different types of tumor cells as well as in nearly identical cell lines derived from different sources.

Profiling protease specificity using a library of inhibitors.

[0174] To take advantage of the flexibility and ease of synthesis of the DCG-04 family of compounds we created a small library of compounds in which the peptide 15 recognition portion of the molecule is modified. It has been proposed that the main specificity regions within the active binding site of the cathepsins are S2, S1, S1', and S2', with S2 containing the main binding pocket (Turk *et al.* (1998) *Biol. Chem.*, 379: 137-147). Since the leucine residue of E-64 binds in the critical S2 pocket of many proteases (Matsumoto *et al.* (1999) *Biopolymers* 51: 99-107), changes to this residue are likely to 20 have the greatest effect on specificity of our inhibitor for a given target. A complete scanning library consisting of 18 natural amino acids and the isosteric methionine analog norleucine was constructed. This library of inhibitors was used to create profiles of inhibitor specificity for proteases targeted by DCG-04 and MB-074 (Figure 6).

[0175] Competition analysis was used to determine the potency of each member of 25 the P2 scanning library towards multiple protease targets. Lysates from DC2.4 cells were pre-incubated with 50μM of each of the 19 DCG library members and residual activity measured for multiple proteases using ¹²⁵I-DCG-04 (Figure 6A). In general, residues containing non-charged aliphatic side chains, isoleucine (I), leucine (L; DCG-04) and norleucine (n), show highest activity and the lowest amount of specificity across the 30 profile of polypeptides. More interesting was the apparent selectivity of several DCG family compounds for a subset of labeled polypeptides. For example, the valine

containing compound competed for polypeptides 1, 2 and cathepsin B but had little effect on the remaining species. In contrast, both the phenylalanine and tyrosine containing compounds showed specificity for polypeptides 2, 3, 4, and 5. Furthermore, while the aspartic acid and glycine containing compounds showed relatively poor activity overall, 5 they showed some degree of specificity against polypeptide 2. Using this data to simultaneously score inhibitors for potency and selectivity will be valuable for the development of specific inhibitors.

[0176] Similar competition experiments were performed with the library of DCG analogs to obtain profiles of single proteases. DC2.4 lysates were preincubated with P2 10 library and then labeled with the cathepsin B-specific compound ^{125}I -MB-074 (Figure 6B). This method allowed analysis of cathepsin B specificity in crude extracts. As found in the ^{125}I -DCG-04 labeling (Fig. 6B), isoleucine, leucine, valine and norleucine analogs showed the highest activity followed by the aromatic amino acids (W, Y, F) containing 15 compounds. In order to explore specificity profiles for additional cysteine proteases that could not be specifically labeled in crude extracts, we performed the same competition labeling experiment described above using a purified enzyme. Pre-incubation of purified cathepsin H with the library of compounds followed by ^{125}I -DCG-04 labeling resulted in a specificity profile that was remarkably similar to the profile observed for cathepsin B in 20 crude extracts (Fig. 6C). While these two proteases are quite different in their biological functions, it is clear from these data that the two have similar inhibitor specificity in the S2 pocket.

[0177] Since it is unlikely that two distinct proteases will exhibit identical reactivity across a diverse set of inhibitors, it may be possible to use this information from 25 positional scanning inhibitor libraries to generate "specificity fingerprints" for a series of well characterized proteases. Establishment of a database of protease inhibitor profiles could potentially be used to establish target identification by labeling of crude protein mixtures in the presence of compound libraries. Furthermore, extension of this methodology to longer, more diverse peptide substrate analogs may further accentuate the specificity differences of closely related protease species.

Profiling across tissue types.

[0178] Having determined that both DCG-03 and DCG-04 were capable of covalently modifying multiple papain family proteases in extracts generated from several cell lines, we wanted to test the utility of these reagents for profiling protease activity 5 patterns in various tissues. In this way, a crude map of protease activities can be created for each tissue and ultimately the identity of these major species can be determined by virtue of their reactivity towards the DCG-04 affinity probe.

[0179] Samples of rat brain, kidney, liver, prostate and testis tissue were used to make crude homogenates at the reduced pH of the lysosome (pH 5.5). Samples were 10 labeled with ¹²⁵I-DCG-04 and analyzed by SDS-PAGE/autoradiography (Figure 7). The most intense labeling in the 20-30 kDa size range was observed for kidney and liver tissue consistent with the known protein processing functions of these organs. Comparison of the labeling profiles across tissue samples indicated that while some of the modified polypeptides were observed in multiple tissues at nearly identical intensities, several 15 polypeptides showed increased or specific activity in a given tissue type. These data are consistent with the findings that cathepsin expression patterns and activities are differentially regulated across tissue types (Kominami *et al.* (1985) *J. Biochem.* 98: 87-93). In addition, the major species labeled by ¹²⁵I-DCG-04 were in the 20-30 kDa size range and are likely to be lysosomal cathepsins such cathepsin B, H and L. To confirm 20 this hypothesis we chose rat kidney as a starting material for the affinity purification of targeted cysteine protease using DCG-04 as an affinity tag. The results of this purification are described below.

Identification of DCG-04 modified proteins in rat kidney by affinity

chromatography.

25 [0180] Perhaps the greatest attribute of a functional proteomics tool is its ability to aid in the identification of targeted proteins. As shown above, rat kidney contains several polypeptides that were efficiently targeted by DCG-04 (Figure 7). Three prominently labeled species of 23kD, 28kD, and 30kD were identified in total kidney extract (Figure 8A). When subjected to anion exchange chromatography, these polypeptides partitioned 30 over a wide range of the elution gradient as determined by DCG-04 labeling of column

fractions (Fig. 8B). Two pools of fractions were chosen based on differences in labeled protein composition. Fractions 7-9 contained predominantly the 23 and 28 kDa species and fractions 11-13 contained the 23, 28 and 30 kDa species. Modified proteins were affinity purified using a monomeric-avidin column that has a reduced binding affinity for 5 biotin and thus the bound proteins could be competitively eluted with high concentrations of biotin (2 mM). The affinity column purified all DCG-04 modified polypeptides in both pools as visualized by SDS-PAGE and silver staining of eluted fractions (Figure 8C). To further resolve DCG-04 modified polypeptides, peak fractions were concentrated, separated by 2D SDS-PAGE and visualized by silver staining (Figure 8D).

10 [0181] The 30 kDa polypeptide (cat B) yielded a single spot near the acidic end of the gel, while the 28 kDa polypeptide (spot #1) resolved into a streak near the basic end of the gel. The 23 kDa band yielded three distinct spots ranging in pI from acidic to basic (spots #2-5). All spots were excised from the gel and subjected to in-gel trypsin digestion, followed by peptide extraction and analysis by mass spectrometry. The protein amount in 15 the 30 kDa spot was not sufficient for unambiguous identification based on MS data alone. Thus its identity was confirmed as cathepsin B by labeling of anion exchange column fractions with the cathepsin B specific label ¹²⁵I-MB-074 (Bogyo *et al.* (2000) *Chem Biol.*, 7: 27-38) (data not shown).

20 [0182] The tryptic mass fingerprint obtained for the 28 kDa band as well as two of the three 23 kDa spots (#2, #3) indicated the presence of cathepsin H. Furthermore, all three digests contained a MH⁺ 1429.7 peptide that was sequenced by low energy dissociation analysis (CID; Figure 9). The resulting sequence, MGEDSYPPYL/IGK (SEQ ID NO:2), unequivocally matched cathepsin H. The amino terminus of cathepsin H is heterogeneous, explaining the presence of multiple cathepsin H isoforms at similar 25 molecular weights (Ishidoh *et al.* (1998) *Biochem. Biophys. Res. Comm.* 252: 202-207). In addition cathepsin H exists as both single chain and two-chain isoforms differing by about 5kDa (Ishidoh *et al.* (1998) *Biochem. Biophys. Res. Comm.* 252: 202-207). Thus, spot #1 is likely to be the single chain form of cat H while spots 2 and 3 may represent heavy chain versions of the two-chain isoform.

30 [0183] The remaining 23kDa spots (#4, #5) did not yield sequence data, however spot #5 was identified as cathepsin L based on the tryptic peptides observed in its digest, its size and pI. Thus, DCG-04 successfully identified the predominant active cysteine

proteases in rat kidney as cathepsin B, H, and L in agreement with previous studies (Kominami *et al.* (1985) *J. Biochem.* 98: 87-93).

Conclusion:

[0184] The need for functional proteomics methods is becoming more important as 5 genomics efforts complete the sequences of various organisms. Cravatt and co-workers have established the utility of a functional proteomics tool specific for the serine hydrolase family of proteases (Liu *et al.* (1999) *Proc. Natl. Acad. Sci., USA*, 96: 14694-14699). We show here that a general affinity label, DCG-04 and its radiolabeled counterpart ¹²⁵I-DCG- 10 04 can be used to profile cysteine protease activities in crude extracts from cells and tissues, as well as throughout multiple stages of a physiological process. Diversification of the peptide portion of the inhibitor using solid-phase synthesis established the utility of small libraries of compounds for determining profiles of inhibitor specificity for both 15 characterized and potentially novel enzymes. The information obtained from these libraries provides a starting point for the development of protease-specific inhibitors and also provides functional information about a protease target that may serve as a method for rapid identification of targets in crude protein mixtures. Furthermore DCG-04 can be used 20 as an affinity purification reagent to aid in the identification of proteases selected by virtue of their reactivity towards our electrophilic probes. Target identification of proteases from crude extracts based on activity profiles will assist in the assignment of protein function as well as potentially identify new players in processes such as carcinogenesis. Finally, further diversification of these reagents is likely to extend their utility for the study of additional physiological processes that are regulated by proteolysis.

Experimental Procedures:

Synthesis of DCG-04, DCG-03, and P2 diverse library.

25 **Solution Phase Synthesis of ethyl (2S,3S)-oxirane-2,3-dicarboxylate.**

[0185] The synthesis of this compound was according to the method described by Bogyo *et al.* (2000) *Chem Biol.*, 7: 27-38.

Solid-Phase Synthesis of DCG-04 and DCG-03.

[0186] The details of the solid-phase synthesis are shown in Figures 2A and 2B. All resins and reagents were purchased from Advanced Chemtech (Louisville, KY). Dry Fmoc-Rink amide resin (0.7mmol/gram) was weighed into 1X10 cm columns (Waters).

5 The columns were fitted with Teflon stopcocks and connected to a 20 port vacuum manifold (Waters) that was used to drain solvents and reagents from the columns. The resin was swelled using DMF. The Fmoc protecting group was removed (deprotected) by treatment with a 20% piperidine solution in DMF for 15 min. The resin was washed with 3 X 3mL of DMF and 3 X 3mL of CH_2Cl_2 .

10 [0187] Fmoc-Lys(biotin)-OH (100 mg, 70 μmol , 1eq), DIC (11.4 μl , 112 μmol , 15 eq), HOBT(15.1mg, 112 μmol , 1.5 eq) were dissolved in 2 ml of DMF, added to the resin and the reaction was agitated for 1 hour. The resin was washed and the N-terminal Fmoc group was deprotected. Fmoc-6-aminohexanoic acid (74.2mg, 210 μmol , 3 eq), DIC (21.4 μl , 210 μmol , 3 eq) and HOBT (28.4mg, 210 μmol , 3 eq) were dissolved in 2 ml DMF

15 and agitated with the resin for 1 hour, followed by washing and deprotection of the N-terminal Fmoc group (synthesis of DCG-03 leaves this step out). Fmoc-Tyr(But)-OH (160.8mg, 350 μmol , 5 eq), DIC (35.6 μl , 350 μmol , 5 eq), and HOBT (47.2mg, 350 μmol , 5 eq) were dissolved in 2 ml DMF and the reaction agitated for 1 hour followed by washing and N-terminal Fmoc group deprotection. Fmoc-Leucine (61.8mg, 350 μmol , 5 eq), DIC (35.6 μl , 350 μmol , 5 eq), and HOBT (47.2mg, 350 μmol , 5 eq) were dissolved in 2 ml

20 DMF and the reaction agitated for 1 hour. The resin was washed followed by deprotection of the N-terminal Fmoc group. Ethyl (2S,3S)-oxirane-2,3-dicarboxylate (22.4mg, 140 μmol , 2 eq), DIC (14.2 μl , 140 μmol , 2 eq), and HOBT (18.9mg, 140 μmol , 2 eq) were dissolved in 2 ml DMF and the reaction agitated for 1 hour. The resin was washed with 25 3X3 ml of DMF and 3X3 ml of CH_2Cl_2 .

[0188] The inhibitors were cleaved from the resin using 1 mL of cleavage cocktail (95% TFA, 2.5% water, 2.5% triisopropylsilane). The mix was collected and the resin washed with 0.5 mL of fresh cleavage cocktail. Ice cold ether (15 ml) was used to precipitate the product. The solid was collected and dissolved in a minimal amount of

30 DMSO. The product was purified on a C18 reverse phase HPLC column (Waters, Delta-Pak) using a linear gradient of 0-100% water-acetonitrile. Fractions containing the product were pooled, frozen and lyophilized to dryness. The identity of the product was

confirmed by mass spectrometry. Electrospray mass spectrum: [M+H] calc'd for DCG-03 C₃₇H₅₅N₇O₁₀S 791.0 found 791.0; calc'd for DCG-04 C₄₃H₆₆N₈O₁₁S 903.1 found 903.7.

[0189] A similar protocol was used to synthesize the P2 diverse library except that synthesis was performed using a 96 well manifold (Robbins Scientific). Synthesis was 5 carried out on 20 mg of Rink resin per well and all coupling conditions were identical to those described above. Each of 18 natural amino acids (excepting cysteine and methionine) and including norleucine were coupled after addition of the amino hexanoic acid spacer group. All subsequent steps were performed as described above except peptides were used without HPLC purification due to the fact that products were found to be pure by HPLC analysis. Identity of products was confirmed by mass spectrometry. 10 Electrospray mass spectrum : X=Ala calc'd [M+H] for C₄₀H₆₀N₈O₁₁S 862.0 found 861.9; Arg C₄₂H₆₆N₁₂O₁₁S 946.5 found 946.7; Asn C₄₁H₆₁N₉O₁₂S 905.0 found 904.9; Asp C₄₁H₆₀N₈O₁₃S 906.0 found 905.9; Glu C₄₂H₆₂N₈O₁₃S 920.0 found 919.8; Gln C₄₂H₆₃N₉O₁₂S 919.0 found 918.9; Gly C₃₉H₅₈N₈O₁₁S 848.0 found 847.7; His 15 C₄₃H₆₂N₁₀O₁₁S 928.0 found 927.7; Ile C₄₃H₆₆N₈O₁₁S 904.1 found 904.0; Leu C₄₃H₆₆N₈O₁₁S 904.1 found 904.0; Lys C₄₃H₆₇N₉O₁₁S 919.0 found 919.0; C₄₆H₆₄N₈O₁₁S 938.0 found 937.8; Pro C₄₂H₆₂N₈O₁₁S 888.0 found 877.8; Ser C₄₀H₆₀N₈O₁₂S 878.0 found 877.8; Thr C₄₁H₆₂N₈O₁₂S 892.0 found 892.0; Trp C₄₈H₆₅N₉O₁₁S 977.1 found 976.7; Tyr C₄₆H₆₄N₈O₁₂S 954.1 found 953.8; Val C₄₂H₆₄N₈O₁₁S 890.0 found 890.0; Nle 20 C₄₃H₆₆N₈O₁₁S 904.1 found 903.9.

Radiolabeling of inhibitors.

[0190] All compounds were iodinated and isolated using the protocol described by Bogyo *et al.* (2000) *Chem Biol.*, 7: 27-38.

Preparation of cell and tissue lysates.

25 [0191] Tissues were dounce-homogenized in buffer A (50 mM Tris pH 5.5, 1 mM DTT, 5 mM MgCl₂, 250 mM sucrose) and extracts centrifuged at 1,100 x g for 10 min at 4°C. The resulting supernatant was centrifuged at 22,000 x g for 30 min at 4°C and final supernatants used for all labeling experiments. Cells were lysed using glass beads (<104 microns) in buffer A and supernatants centrifuged for 15,000xg for 15 min at 4°C. The

total protein concentration of the final supernatants (soluble) was determined by BCA protein quantification (Pierce).

Labeling of lysates with ^{125}I -DCG-04, ^{125}I -DCG-03, and ^{125}I -MB-074.

5 [0192] Equivalent amounts of radioactive inhibitor stock solutions (approx. 10^6 cpm per sample) were used for all labeling experiments. Samples of lysates (100 μg total protein in 100 μL buffer; 50 mM Tris pH 5.5, 5 mM MgCl₂, 2 mM DTT) were labeled for 1 hour at 25°C unless noted otherwise. Samples were quenched by dilution of 4X SDS sample buffer to 1X (for 1D SDS-PAGE) or by dissolving urea to a final concentration of 9.5 M (for 2D SDS-PAGE).

10 **Gel electrophoresis.**

15 [0193] One-dimensional SDS-PAGE, two-dimensional IEF gels were performed as described (Bogyo *et al.* (1998) *Chem Biol.*, 5: 307-320).

SDS/PAGE-western blotting detection of and auto-radiography of DCG-04 modified proteins.

20 15 [0194] Quenched DCG-04 labeled samples were separated by SDS/PAGE (100 $\mu\text{g}/\text{lane}$) and transferred to nitrocellulose using semi-dry apparatus. Membranes were blocked using phosphate buffered saline (PBS) and 5% (w/v) dry milk for 30 min at 25°C. Blots were washed briefly with PBS/0.2% Tween (PBS-Tween) and treated with avidin-horseradish peroxidase conjugate (VectaStain) in PBS-Tween for 30 min. at 25°C. Blots were washed three times with PBS-Tween, treated with ECL reagents (Amersham), and exposed to film.

Competition labeling experiments.

25 [0195] Lysates from the dendritic cell line DC2.4 were prepared at pH 5.5 as described above. Purified cathepsin H was purchased from Calbiochem (San Diego, CA). Samples of lysates (100 μg total protein in 100 μL buffer B; 50 mM Tris pH 5.5, 5 mM MgCl₂, 2 mM DTT) or purified cathepsin H (1 μg protein in 100 μL buffer A) were preincubated with 50 μM of each library member (diluted from 5 mM DMSO stocks) for 2 hrs at room temperature. Samples were then labeled by addition of either ^{125}I -DCG-04 or

¹²⁵I-MB-074 to each sample followed by further incubation at room temperature for 1 hour. Samples were quenched by the addition of 4 X sample buffer to 1X followed by boiling for 5 minutes. Samples were analyzed by SDS-PAGE followed by autoradiography.

5 Preparation of mouse carcinoma cell lines.

[0196] Mouse melanoma cell lines were prepared by a single topical application of 25 µg of the chemical mutagen dimethylbenzanthracene (DMBA) to the skin of mice followed by biweekly application of 100µM of the tumor promoter, TPA, over an extended period of time essentially as described (Bremner and Balmain (1990) *Cell* 61: 10 407-417; Burns *et al.* (1991) *Oncogene* 6: 2363-2369; Haddow *et al.* (1991) [published erratum appears in *Oncogene* 1991 Dec;6(12):2377-8]. *Oncogene* 6: 1465-1470).

Protein identification of DCG-04 modified proteins.

[0197] A soluble fraction of rat kidney lysate (80 mg total protein) was diluted into anion exchange starting buffer (50mM Tris, 50mM NaCl, pH 9.0). The lysate was applied 15 to a HitrapQ anion exchange column (Amersham Pharmacia Biotech) and eluted using a linear gradient of 0.05-1M NaCl, pH 9. An aliquot from each fraction (50 µL) was incubated with 50µM DCG-04 at 25°C for 1hr and analyzed on a 12.5% SDS/PAGE gel followed by affinity blotting as described above.

[0198] The fractions containing peak labeling of the 25kD-30kD bands were 20 pooled and DCG-04 was added to a final concentration of 50 µM. Pools were incubated at 25°C for 2 hours and then 12 hours at 4°C. Unbound inhibitor was removed and buffer was exchanged with PBS using a PD-10 column (Pharmacia). Samples were applied to a monomeric-avidin column (1 ml bed volume; Pierce) and the column was washed with 6X 1ml fractions of 1M NaCl. Bound proteins were eluted with 0.5 ml fractions of 2mM 25 Biotin/100mM NH₄HCO₃ buffer. All wash and eluent fractions were analyzed by SDS/PAGE and silver staining. The fractions containing the labeled 25-30kD bands were pooled, the volume reduced by lyophilization and solid urea added to 9.5 M along with BME to 5%, NP-40 to 2%, pH 5-7 ampholytes to 1.6% and pH 3.5-10 ampholytes to 30 0.4%. Samples were applied to IEF tube gels and electrophoresed at 1000V for 13 hours followed by separation in the second dimension on 12.5% SDS-PAGE gels.

[0199] The resulting gels were fixed in 12% acetic acid/50%methanol stained with silver according to reported protocols (Bogyo *et al.* (1998) *Chem Biol.*, 5: 307-320). Spots were excised, digested with trypsin, and the peptide molecular weight measurements were carried out by MALDI-MS (PE Voyager DESTR). Sequence determination was 5 performed on a quadrupole time-of-flight hybrid tandem mass spectrometer (PE QSTAR) equipped with a Protome nanospray source. This instrument affords high resolution and accuracy for mass measurement and the CID data obtained allowed unambiguous sequence determination. Database searches were performed using the Protein Prospector software package (<http://prospector.ucsf.edu/>).

10

Example 2

Chemical approaches for functionally probing the proteome

Introduction.

[0200] Over the past few years the complete genome sequences of multiple organisms have been determined. These efforts have been followed by the annotation of 15 genes that code for all proteins of an organism's proteome. While this information is likely to provide valuable information, a great deal of effort is required to define the function of individual gene products. Informatics techniques have been developed to assign function to individual genes by analyzing patterns of co-inheritance throughout multiple organisms (Marcotte *et al.* (1999) *Nature* 402: 83-86; Eisenberg *et al.* (2000) 20 *Nature* 405: 823-826). Furthermore, analysis of genome-wide changes in transcription in response to different stimuli allows clustering of genes of similar function based on transcriptional co-regulation (Eisen *et al.* (1998) *Proc. Natl. Acad. Sci., USA*, 95: 14863-14868). While these methods help to broadly classify proteins into families based on 25 predicted function, the assignment of functions to specific members within a large enzyme family remains a difficult task.

[0201] Proteomics approaches address some of the gaps in genomics methodologies by profiling and identifying bulk changes in protein levels (Dove (1999) *Nat Biotechnol* 17: 233-236; Pandey and Mann (2000) *Nature* 405: 837-846). However, 30 these methodologies only provide information for abundant proteins while proteins with difficult biochemical properties (i.e. membrane proteins) are often excluded from

analysis. Moreover, for most enzymes, their activity, and therefore their function, is regulated by a complex set of post-translational controls. Therefore, even proteomic profiles in many cases provide an incomplete picture of how enzymes are functionally regulated (Gygi *et al.* (1999) *Molecular and Cellular Biology* 19: 1720-1730).

5 [0202] Classical genetic approaches are tried and true methods to assign functions to specific gene products. In many biological systems it is possible to disrupt a desired gene and assess the resulting phenotype. However, this process is often tedious and in cases where multiple related proteins have similar functions, compensation adjustments make the resulting phenotype difficult to interpret.

10 [0203] To circumvent these problems, small molecules can be used to manipulate the activity of protein targets (Stockwell (2000) *Trends Biotechnol* 18: 449-455; Schreiber (1998) *Bioorg Med Chem* 6: 1127-1152). This "chemical genetic" approach makes use of libraries of small molecules to screen for compounds that perturb a given biological process. The resulting 'hits' can then be used to begin to assign function to specific

15 enzyme or protein targets. However, the utility of this process is limited by the difficult task of identifying the relevant target of the small molecule.

[0204] In the case of traditional drug discovery, small molecule libraries are screened against a single pre-defined target. Lead compounds are often identified from large chemical libraries using an *in vitro* assay. While many of these compounds are

20 effective against the purified target, little is usually known about their selectivity in a crude proteome. Therefore, a method that allows screening for small molecule inhibitors in cell and tissue extracts or intact cells would allow identification of lead compounds based on multiple criteria such as potency, selectivity and cell permeability. Furthermore, compounds could be screened against entire enzyme families thereby increasing the

25 chances of identifying useful compounds for therapeutic intervention.

[0205] We have developed chemically reactive affinity probes that can be used to (i) identify the members of a given enzyme family within a proteome (ii) determine the relative activity levels of individual family members (iii) localize active enzymes within a cell (iv) screen small molecule libraries directly in crude protein extracts for inhibitors that

30 can ultimately be used to determine biological functions of specific target enzymes. In this study, we have chosen to focus on the papain family of cysteine proteases for several

reasons. Firstly, these proteases are synthesized as inactive zymogens that are activated post-translationally (Cygler *et al.* (1996) *Structure* 4: 405-416; Coulombe *et al.* (1996) *Embo Journal* 15: 5492-5503). Their activity can also be regulated by interaction with macro-molecular inhibitors resulting in transcription/translational profiles that provide

5 only limited information regarding their functional regulation. Secondly, the papain family is composed of many closely related family members whose functions are poorly defined (Chapman *et al.* (1997) *Annu. Rev. Physiol.* 59: 63-88). Thirdly, many small molecule covalent inhibitors of this class of enzyme have been developed that can be used for probe design (see, Shaw (1994) *Meth. Enzymology* 244: 649-656, and refs therein).

10 Finally, these enzymes have been found to play an important role in many disease conditions such as cancer (Yan *et al.* (1998) *Biol. Chem.*, 379: 113-123), osteoporosis (Gelb *et al.* (1996) *Science* 273: 1236-1238), asthma (Chapman *et al.* (1997) *Annu. Rev. Physiol.* 59: 63-88), and rheumatoid arthritis (Iwata *et al.* (1997) *Arthritis and Rheumatism* 40: 499-509) making them a potential important class of enzymes for drug

15 development.

Results and Discussion

Probe design and application to pure enzymes, crude homogenates and intact cells.

[0206] Several laboratories have developed small molecule electrophiles that show class-specific reactivity towards nucleophilic active site residues of several different enzyme families. These include serine (Liu *et al.* (1999) *Proc. Natl. Acad. Sci., USA*, 96: 14694-14699; Kidd *et al.* (2001) *Biochem.*, 40: 4005-4015) and cysteine (Bogoy *et al.* (2000) *Chem Biol* 7: 27-38; Greenbaum *et al.* (2000) *Chem Biol* 7: 569-581; Faleiro *et al.* (1997) *Embo Journal* 16: 2271-2281) hydrolases as well as aldehyde dehydrogenases

20 (Adam *et al.* (2001) *Chem Biol* 8: 81-95). In each case, electrophiles have been designed that exhibit broad irreversible reactivity for enzyme family members, while remaining relatively inert towards free-circulating nucleophiles such as thiols, hydroxyls and amines. The resulting activity-based probes (ABPs) can be used to covalently label specific target enzymes within the complex mixture of proteins from a cell or tissue sample. Our

25 laboratory has developed probes based on the structure of the natural product E-64 19 . These ABPs can be used to affinity label papain family cysteine proteases. They also

30

allow rapid purification of labeled proteases by virtue of incorporation of a biotin affinity tag. Here we have used the core peptide epoxide analog of E-64 to create four fluorescently labeled ABPs for papain family cysteine proteases (Figure 13).

[0207] These probes incorporate four different fluorescent moieties, each with

5 non-overlapping excitation and emission spectra, allowing for multiplexing of probes. Four BODIPY analogs were chosen based on the excitation and emission wavelengths of fluorophores commonly used in DNA sequencing protocols. We reasoned that it should be possible to visualize and quantify fluorescently labeled proteins using a standard DNA sequencing apparatus equipped with a high intensity laser. Figure 14A shows the gel
10 image that results from incubation of eight different purified papain family cysteine proteases with each of the four fluorescent ABPs followed by analysis on an ABI 377 DNA sequencer. Using these probes, it is possible to load all eight proteases in a single gel lane and distinguish each, based on differences in molecular weight and emission wavelength of fluorescent labels.

15 [0208] The same four probes were next used to profile the repertoire of papain family proteases within a complex protein mixture derived from a tissue homogenate.

Figure 14 shows the profiles of cysteine proteases in total rat liver homogenates obtained by labeling with the biotinylated probe DCG-04, the radiolabeled version of DCG-04, and the four fluorescent analogs of DCG-04. All ABPs labeled the same four predominant
20 protease species with only slight differences in relative intensities observed for each probe. These results suggest that the presence of structurally diverse labeling groups at the distal affinity site of the molecules had little effect on a compound's ability to covalently modify its targets.

[0209] Since covalent modification of target proteases by the ABPs requires

25 modification of the active site thiol nucleophile, labeling intensities can be used as an indirect measure of enzymatic activity. Thus, unlike antibodies that can only be used to monitor bulk levels of specific proteins, these reagents allow analysis of changes in levels of enzymatic activity. In the past, our laboratory has used these reagents to follow activity of cysteine proteases during processes such as tumor progression and cell invasion (Bogyo
30 *et al.* (2000) *Chem Biol* 7: 27-38). These newly developed ABPs therefore provide an efficient method for monitoring changes in protease activities within a proteome.

[0210] Since the fluorescent probes are cell permeable they make ideal tools for imaging of protease activity in intact cells or tissue sections. Figure 15 shows the dendritic cell line DC2.4 either directly labeled *in situ* with green-DCG-04 or pre-treated with E-64 and then labeled with the fluorescent probe. Cells directly treated with the 5 green ABP showed a fluorescence staining pattern characteristic of lysosomal compartments. Cells that had been pre-treated with E-64 showed diffuse fluorescence throughout the cytosol, likely due to residual free probe that failed to be washed away. The cells were collected after imaging, lysed and analyzed by SDS-PAGE and fluorescence detection. The resulting profiles indicated that multiple protease species 10 were labeled by the fluorescent probe and that these proteases were completely inhibited by pre-treatment of cells with E-64. Thus the fluorescent staining observed in the non-pretreated cells represents the localization of active papain family cysteine proteases. This method is likely to be applicable to tissue samples and may serve as a convenient way to image protease activities in tissues derived from important clinical samples such as solid 15 tumors.

Using ABPs to generate inhibitor specificity profiles for papain family proteases.

[0211] The concept of classifying enzyme family members based on structure-activity relationship homology (SARAH) has been proposed as an alternative to 20 classification methods based on sequence homology (Frye (1999) *Chem Biol* 6: R3-7). Using this approach, large enzyme families can be classified based on their reactivity towards small molecule ligands thereby aiding the process of functional analysis and drug design. ABPs serve as ideal tools for the rapid analysis of SARAH between closely 25 related enzyme family members. Furthermore, ABPs allow SARAH analysis directly in crude protein extracts thereby allowing the classification of potentially novel target enzymes.

[0212] To begin SARAH analysis of papain family enzymes, a series of small molecule libraries were designed based on a core peptide backbone coupled to the epoxide electrophile contained in the DCG-04 probes (Figure 16A). Initially, positional scanning 30 libraries (PSLs) were synthesized in which a single amino acid position was scanned through a series of natural and non-natural amino acids, while the remaining two positions

were coupled with a mixture of all possible natural amino acids (minus cysteine and methionine and including norleucine). The resulting sub-libraries were composed of 361 members each. Scanning of constant amino acids at the P3 and P4 positions through all natural amino acids indicated that these elements did not significantly contribute to

5 selectivity of inhibitor binding (data not shown). Therefore, only data compiled for scanning of the constant P2 position are presented. To increase the diversity of the small molecules in the PSLs we included 42 hydrophobic non-natural amino acids as building blocks (*see structures in Table 1*). In addition, each of the natural amino acids was coupled to the mirror-image enantiomeric form of the epoxide (2R, 3R vs. 2S, 3S).

10 Previous work indicates that this change in stereochemistry favors binding of the inhibitors on the prime side of the active site resulting in more diversity in our libraries (Schaschke *et al.* (1997) *Bioorganic and Medicinal Chemistry* 5: 1789-1797).

Table 1. Non-natural amino acids used in PSLs.

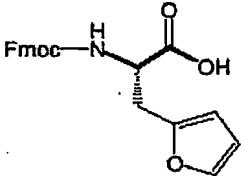
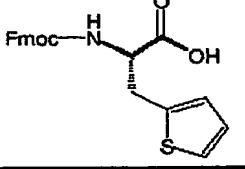
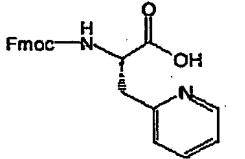
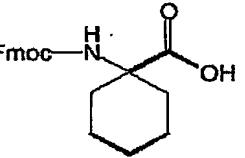
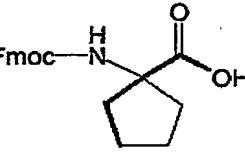
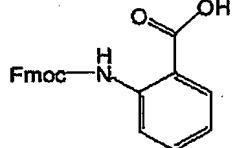
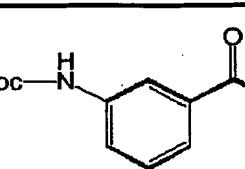
cmpd #	Amino Acid	Structure
1	(2furyl)alanine	
2	(2thienyl)alanine	
3	2pyridylAla	
4	1amino1cyclohexane carboxylic acid	
5	1amino1cyclopentanecarboxylic acid	
6	2-Abz	
7	3Abz	

Table 1 cont'd.

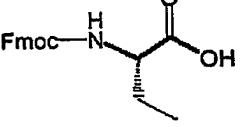
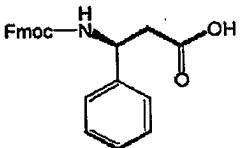
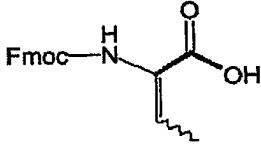
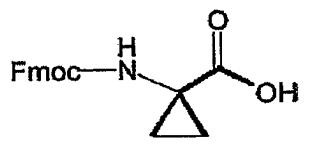
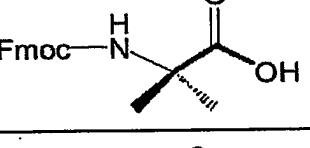
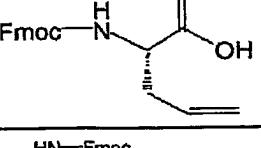
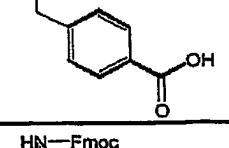
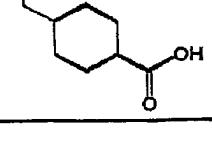
8	2Abu	
9	3amino3phenylpropanoic acid	
10	dehydroAbu	
11	ACPC	
12	Alb	
13	AllylGly	
14	Amb	
15	Amc	

Table 1 cont'd.

5	16	Bip	
10	17	Bpa	
15	18	Cba	
20	19	Cha	
25	20	deltaLeu	
	21	deltaVal	
	22	Hyp	

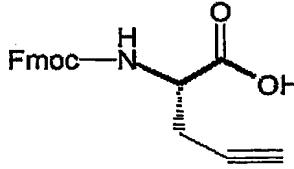
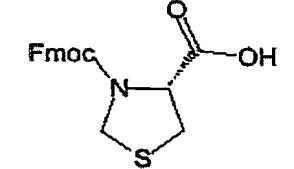
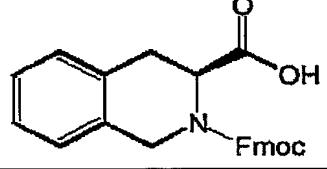
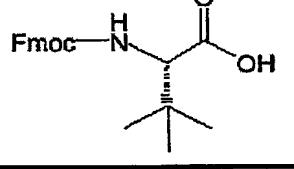
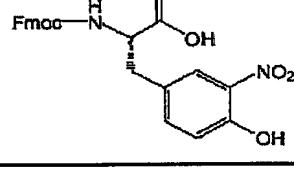
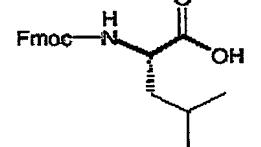
Table 1 cont'd.

5	23	Igl	
10	24	Inp	
15	25	1-Nal	
20	26	2-Nal	
25	27	Nva	
	28	4-nitroPhe	
	29	4MethylPhe	

Table 1 cont'd.

30	4Methyl-DPhe	
31	Phe(pl)	
32	Phe4NH(Boc)	
33	hPhe	
34	Phg	
35	plp	
36	Dpip	

Table 1 cont'd.

37	propargylglycine	
38	Thz	
39	Tic	
40	Tle	
41	3-NitroTyr	
42	Ieu	

[0213] PSLs were first screened against thirteen purified papaine family enzymes (Figure 16B). Potency was assessed by pretreatment of pure enzymes with each library followed by labeling with ^{125}I -DCG-04 and analysis by SDS-PAGE and autoradiography. The ability of each library to block active site labeling by DCG-04 was measured as the percentage of competition relative to an untreated control. The resulting values were

visualized using software developed by Eisen and co-workers designed to analyze data generated from micro-array analysis (Eisen *et al.* (1998) *Proc. Natl. Acad. Sci., USA*, 95: 14863-14868). This software assigns a color to numerical competition values and allows clustering of profiles based on similarities across diversity positions (X-axis) and enzyme family members (Y-axis). The resulting "clustergram" is shown in figure 16B.

5 [0214] Clustering data throughout the constant amino acid residues grouped the data, such that residues that showed overall poor binding to all targets were positioned to the right and residues that showed universal strong binding were positioned to the left. The remaining residues in the middle of the clustergram showed some degree of 10 selectivity for individual enzymes. The results from the clustering indicate that the non-natural amino acids and natural amino acids linked to the (R,R) enantiomer of the epoxide provided the greatest target selectivity.

15 [0215] Similarly, clustering the data across the Y-axis grouped the enzymes based on similarities in specificity fingerprints or SARAHs. The clustering therefore allowed enzymes to be classified based on active site topology, as reflected in their ability to bind sets of small molecule ligands. The results from the cluster indicate that, in general, enzymes that are closely related by sequence homology (i.e. Cat V and Cat L) tend to cluster together based on specificity fingerprints. However, in some cases enzymes with close sequence homology showed markedly different specificity profiles with respect to 20 inhibitor/substrate binding (i.e. Cat C and Cat B). Thus, specificity profiling provides a potentially more informative means for grouping related enzymes. Furthermore, it is possible to classify unknown protease species from crude extracts by generating fingerprints of targets and comparing them to fingerprints of well-characterized family members. This technique allows rapid classification of unknown enzymes and provides 25 useful information for the design of small molecule inhibitors targeted for them.

Using ABPs to screen for selective inhibitors of papain family cysteine proteases in crude tissue extracts.

30 [0216] Perhaps the most powerful attribute of ABPs is their ability to facilitate screening of small molecule inhibitors against complete enzyme families without the need to first identify, clone and express individual targets. Furthermore, the data that is obtained from the screening process provides information not only regarding potency of

the potential lead compounds, but also regarding selectivity of the compounds in a physiologically relevant sample that contains many closely related family members. To demonstrate the utility of this approach we performed screening of our PSLs in crude liver extracts (Figure 16C). Specificity profiles for each of the major protease species labeled by DCG-04 were obtained. The resulting clustergram indicated that several residues which clustered to the center of the profile could be selected that would confer unique specificity for an individual protease species in the extract. Therefore, this method yielded interesting lead compounds using a relatively small number of libraries (~80) with limited structural diversity. A similar screen of a larger, more structurally diverse small molecule library is likely to provide an even greater number of inhibitor leads. Given the relative ease of screening and the abundance of the protein extracts, such a large-scale screen is clearly accessible using this methodology.

Profiling changes in protease activities upon addition of selective small molecule inhibitors.

15 [0217] Analysis of the library data from screening of liver extracts indicated that several PSLs showed selective binding to a single protease. We chose to focus on the constant P2 glutamine (R,R) epoxide library because of its high degree of selectivity for protease #2 in the extract. Liver extracts were either directly labeled with the red-DCG-04 probe or treated with the library and then labeled with the blue-DCG-04 probe. The 20 samples were then combined and subjected to a first dimension of isoelectric focusing followed by analysis by SDS-PAGE in the second dimension using the DNA sequencer (Figure 17A). This method allowed analysis of multiple channels of data in a single gel that could be merged to determine changes in activity of each protease species in the presence of the inhibitor library. The resulting 2D profile unambiguously demonstrated that the glutamine (R,R) library specifically binds to the active site of a single protease 25 (spot #2) as indicated by loss of labeling in the blue channel.

30 [0218] To determine the identity of the protease selectively targeted by the small molecule library, we used the biotin tagged DCG-04 to perform a single-step affinity purification of all labeled proteases from liver extracts. The resulting silver stained 2D profile shows that all fluorescently labeled protease could be rapidly purified from the crude extract and correlated with the labeling profiles (Figure 14B). The silver stained

spot corresponding to spot #2 was excised and identified as cathepsin B by LC-MS-TOF CID sequencing. Furthermore, several other cathepsin family members including cathepsins Z, H, C and J were identified by this method.

Design of selective inhibitors based on library screening data.

5 [0219] Using information from the scanning of our PSLs, we synthesized several single compounds designed to validate the library approach. In all cases a P3 tyrosine was included as a site for radio-iodination and the P2 residue was chosen based on target selectivity. P2 glutamine attached to the (R,R) epoxide inhibitor (YQ-(R,R)-Eps) was chosen because of its selectivity for cathepsin B in the extract and P2 glycine was chosen
10 as a negative control. The cathepsin B specific ABP MB-074 (Bogyo *et al.* (2000) *Chem Biol* 7: 27-38) was used as a control for comparison with YQ-(R,R)-Eps. Compounds were added to extracts over a wide concentration range and activity for each target was assessed by labeling with ^{125}I -DCG-04 (Figure 18A). As expected, YQ-(R,R)-Eps and MB-074 selectively blocked labeling of the cathepsin B band (#2) while GR-(R,R)-Eps
15 showed little or no inhibition of all of the proteases. The newly developed cathepsin B inhibitor was also radioiodinated and used to label liver homogenates (Figure 18B). The labeling profile was compared to the profiles for the cathepsin B-specific probe ^{125}I -MB-074 and the generally reactive probe ^{125}I -DCG-04. YQ-(R,R)-Eps, like MB-074, showed selective labeling of the band identified as cathepsin B. We conclude that it is possible to
20 rapidly identify a structurally distinct class of cathepsin B selective inhibitors by screening of libraries of limited complexity. The resulting lead compound, while not excessively potent, now serves as a template for the design of optimized inhibitors that are distinct from the CA-074 class of cell impermeable cathepsin B inhibitors. No doubt this approach could also be used to selectively target other cathepsin family members through
25 a more extensive library screening effort.

Conclusions.

[0220] In summary, we have developed tools to identify families of related enzymes within a complex proteome. These tools can be used to determine relative activity levels of these enzymes and to visualize their localization in live cells. These
30 tools also allow rapid design and screening of small molecule inhibitors for select targets. In the current study we successfully identified a new cathepsin B selective inhibitor by

screening of a small set of libraries in crude liver extracts. Furthermore, we have developed a general method for rapid analysis of large data sets generated from library screening of multiple targets in crude cell extracts. This approach allows rapid comparison of inhibitors as well as targets based on similarities in structure-function relationships. This general functional proteomic method, although applied here to papain family proteases, can also be used for a wide range of enzyme families through design and synthesis of new families of class-specific affinity probes.

Materials and Methods

Synthesis Protocols

10 **Synthesis of ethyl (2S,3S)-oxirane-2,3-dicarboxylate and ethyl (2R,3R)-oxirane-2,3- dicarboxylate and DCG-04.**

[0221] The synthesis of (2R,3R)-oxirane-2,3-dicarboxylate is identical to that reported for the (2S,3S) isomer (Bogyo *et al.* (2000) *Chem Biol* 7: 27-38). The synthesis of DCG-04 is reported in Greenbaum *et al.* (2000) *Chem Biol* 7: 569-581.

15 **Synthesis of BODIPY558/568-DCG-04, BODIPY 588/616-DCG-04, BODIPY530/550-DCG-04, and BODIPY493/503-DCG-04.**

[0222] All fluorophores where purchased from Molecular Probes (Eugene, OR). A free amino version of DCG-04 was synthesized by replacing the terminal biotinylated lysine with lysine using the reported synthesis protocols for DCG-04 (Greenbaum *et al.* (2000) *Chem Biol* 7: 569-581). Free amino DCG-04 (6 mg, 8.8 mmol, 1.5 eq) and either BODIPY558/56-OSu (3.0 mg, 6.0 mmol, 1.0 eq), BODIPY 588/616-OSu (1.0 eq), BODIPY530/550-OSu (1.0eq), or BODIPY493/503-OSu (1 eq) were dissolved in 100 ml DMSO. Diisopropylethylamine was then added (12.0 mmol 2.0 eq). The reaction was monitored by high performance liquid chromatography (HPLC). After 2 hours the product was purified on a *cis* reverse phase HPLC column (Waters, Delta Pak) using a linear gradient of 0-100% water-acetonitrile. Fractions were pooled and lyophilized to dryness. The identity of the product was confirmed by mass spectrometry. Electrospray mass spectrum: [M+H] calculated for BODIPY558/568-DCG-04 C₄₉H₆₉BF₂N₈O₁₀ 979.5 ,found 978.5, BODIPY 588/616-DCG-04 C₆₀H₇₆BF₂N₉O₁₂S 1196.5 found 1197.0,

BODIPY530/550-DCG-04 $C_{57}H_{69}BF_2N_8O_{10}$ 1075.5 found 1075.0, and BODIPY493/503-DCG-04 $C_{49}H_{63}BF_2N_8O_{10}$ S 1005.4, found 1004.5.

Synthesis of Positional Scanning Libraries.

[0223] Synthesis of the P2 constant PSL library was performed using a 96 well manifold (FlexChem, Robbins Scientific). Each library was constructed using a constant amino acid at the P2 position and an isokinetic mixture of all natural amino acids (minus cysteine and methionine plus norleucine) at the variable position. The isokinetic mixture was created using a ratio of equivalents of amino acids based on their reported coupling rates (Ostresh *et al.* (1994) *Biopolymers* 34: 1681-1689). The total mixture was adjusted to ten-fold excess total amino acids over resin load. For constant positions, a single amino acid was coupled using ten-fold excess. In addition to the natural amino acids, a set of 42 non-natural hydrophobic amino acids were also used for the constant P2 position (see supplemental materials). Couplings were carried out using Diisopropylcarbodiimide (DIC) and Hydroxybenzatrazole (HOBT) under standard conditions for solid phase peptide synthesis. Libraries and single components were cleaved from the resin by addition of 90% trifluoroacetic acid 5% water and 5% triisopropyl silane for 2 hours. Cleavage solutions were collected and products precipitated by addition of cold diethyl ether. Solid products were isolated and the crude peptides were dissolved in DMSO (50 mM stock) based on average weights for each mixture. Libraries and single compounds were stored at -20°C and further diluted to 10 mM stock plates for use in experiments.

Synthesis of Y-Q(R,R)Eps and Y-G(R,R)Eps.

[0224] All single component peptide epoxides were synthesized on the solid support using the protocols reported for DCG-04 (Greenbaum *et al.* (2000) *Chem Biol* 7: 569-581). The inhibitors were cleaved from the resin by addition of 90% trifluoroacetic acid 5% water and 5% triisopropyl silane for 2 hours. Ice cold ether (15 ml) was used to precipitate the products. The crude products were purified on a C_{18} reverse phase HPLC column (Waters) using a linear gradient of 0-100% water-acetonitrile. Fractions containing the product were pooled, frozen and lyophilized to dryness. The identity of the product was confirmed by mass spectrometry. Electrospray mass spectrum: [M+H] calculated for: YG-(R,R)Eps $C_{17}H_{21}N_3O_7$ 380.1, found 380.1; YQ-(R,R)Eps $C_{20}H_{26}N_4O_8$ 451.2, found 451.2.

Radiolabeling of inhibitors.

[0225] All compounds were iodinated and isolated using the protocol described by Bogyo *et al.* (2000) *Chem Biol* 7: 27-38.

Preparation of cell and tissue lysates.

5 [0226] Tissues were dounce-homogenized in buffer A (50 mM Tris pH 5.5, 1 mM DTT, 5 mM MgCl₂, 250 mM sucrose) and extracts centrifuged at 1,100 x g for 10 min at 4°C and the supernatant centrifuged at 22,000 x g for 30 min at 4°C. Cells were homogenized using glass beads in buffer A and supernatants centrifuged for 15,000 x g for 15 min at 4°C. The total protein concentration of the final supernatants (soluble) was
10 determined by BCA protein quantification (Pierce).

Labeling of lysates with DCG-04, ¹²⁵I-DCG-04, ¹²⁵I-MB-074, ¹²⁵I-YQ-(R.R)Eps, Yellow-DCG- 04, Blue-DCG-04,Green DCG-04 or Red-DCG-04.

[0227] Lysates (100 mg total protein in 100 μL buffer; 50 mM Tris pH 5.5, 5 mM MgCl₂, 2 mM DTT) were labeled for 1 hour at 25°C unless noted otherwise. DCG-04 was added to a final concentration of 10 mM. Equivalent amounts of all radioactive inhibitor stock solutions (approx. 10⁶ cpm per sample) were used for all labeling experiments. Fluorescent compounds were added to lysates to a final concentration of 0.1 mM. Samples were quenched by addition of 4X SDS sample buffer (for 1D SDS-PAGE) or by addition of solid urea to a final concentration of 9.5 M (for 2D SDS-PAGE). Fluorescent 20 samples were analyzed using an ABI 377 DNA sequencer. Standard 15% SDS-PAGE gels of 0.4 mm thickness were prepared using 15 cm plates provided by the manufacturer. Samples were loaded and electrophoresed for 3-4 hrs at a constant current of 35 mA with voltage limited to 750 V. Gel images were created using the Gene Scan software provided by the manufacturer. In some experiments, fluorescent samples were analyzed by 25 standard SDS-PAGE followed by scanning with a Molecular Dynamics Typhoon laser scanner.

In situ fluorescence labeling.

[0228] Dendritic cells (DC2.4) were plated on a 24-well dish (10⁵ cells/well) embedded with sterile microscope cover slips, in RPMI medium containing 10% FBS.

After 16 hours, cells were washed with 1 ml TC-199 medium and incubated with 1 mM of Bodipy-DCG-04 in TC-199 for 12 hours at 37°C. Cells were washed 3 times with 1 ml TC-199 and incubated for 5 hr in probe-free medium. Subsequently, cells were either lysed in buffer A and analyzed on a 12.5% SDS-PAGE using a fluorescent scanner or

5 viewed under a fluorescent microscope.

Gel electrophoresis.

[0229] One-dimensional SDS-PAGE and two-dimensional IEF was performed as described by Bogyo *et al.* (1998) *Chem Biol* 5: 307-320.

Competition labeling and analysis of data.

10 [0230] Rat liver lysates (100 mg total protein in 100 μ L buffer A; 50 mM Tris pH 5.5, 5 mM MgCl₂, 2 mM DTT) or purified cathepsins (1 μ g protein in 100 μ L buffer A) were pre-incubated with 10 μ M of each library member (diluted from 10 mM DMSO stocks) for 30 min at room temperature. Samples were then labeled by addition of ¹²⁵I-DCG-04 to each sample followed by further incubation at room temperature for 1 hour.

15 Samples were quenched by the addition of 4 X sample buffer, resolved by SDS-PAGE, and analyzed by PhosphorImaging (Molecular Dynamics). Bands corresponding to each labeled protease were quantitated. Inhibitor treated samples were compared to an untreated control sample. Numerical values for percent written by Eisen and co-workers (Eisen *et al.* (1998) *Proc. Natl. Acad. Sci., USA*, 95: 14863-14868). These programs can

20 be obtained from www.microarrays.org.

Purification and identification of affinity labeled proteases from rat liver.

[0231] Protein lysates prepared in buffer A (50 mM Acetate buffer, 5mM DTT, 0.1% Triton X-100) were incubated with 5 mM DCG-04 for 1.5 hours at room temperature. After incubation the protein lysate was passed through a PD10 column pre-equilibrated with buffer B (50 mM Tris-Base 7.4, 150 mM NaCl) and proteins were eluted with the same buffer. SDS was added to eluted proteins to a final concentration of 0.5% and the solution boiled for 10 minutes, diluted 2.5 fold with buffer B (to reduce SDS concentration to 0.2%) and incubated with 100 ml bed volume of pre-washed Streptavidin beads for 1 hour at room temperature. Beads were washed 5 times with buffer B, and

bound proteins were eluted by boiling for 10 minutes in the presence of 100 ml SDS sample buffer. For 2D analysis, samples in SDS sample buffer were diluted 1:1 with IEF sample buffer (9.5 M, 5% BME, 2% NP-40, 1.6% pH 5-7 ampholines and 0.4% pH 3.5-10 ampholines) and pure NP-40 was added (25% of volume of sample). Samples were applied to IEF tube gels and electrophoresed at 1000V for 13 hours followed by separation in the second dimension on 15% SDS-PAGE gels. The resulting gels were fixed in 12% acetic acid/50%methanol stained with silver according to reported protocols (Bogyo *et al.* (1998) *Chem Biol* 5: 307-320). Spots were excised, digested with trypsin, and fractionated by reversed-phase HPLC on an Ultimate system, equipped with a FAMOS auto-injector (LC Packings, San Francisco, CA). Experimental conditions were: 1 mL injection; 75 mmx150 mm PepMap column; solvent A, H₂O with 0.1% formic acid; solvent B, acetonitrile with 0.1% formic acid; gradient, 0-30% B in 40 min at a flow rate of ~250 nL/min. Mass spectrometry detection was performed on a QSTAR quadrupole-orthogonal- acceleration-time-of-flight tandem mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) in information dependent acquisition (IDA) mode: 2 second survey acquisitions were followed by 5 second CID acquisitions, in which the most abundant ion of each survey scan was selected as the precursor. All the singly charged ions as well as some trypsin autolysis products were excluded from the precursor ion selection. The collision energy was optimized and adjusted automatically depending on the charge state and the m/z value of the precursor ions selected. The mass range recorded in survey acquisitions was m/z 300-1400. For CID experiments the lower mass limit was changed to m/z 60. All the data were measured using a two point external calibration. The instrument affords ~8000 resolution and 30 ppm mass accuracy with external calibration in both MS and CID mode. Proteins were identified automatically by Mascot database search using the MS/MS data (Matrix Science Ltd., London, UK).

Example 3

Uses of Fluorescently Labeled Probes

[0232] Figure 16 illustrates the screening of small molecule libraries against the complete set of papain family cysteine proteases in Rat liver. Total protein extracts from rat liver were incubated with positional scanning libraries of small molecules based on the epoxide probe structure. After 30 minutes pre-incubation with inhibitors, samples treated with compounds 1-20 were labeled with Green-DCG-04. Samples treated with

compounds 21-40 were labeled with Blue-DCG-04, and samples treated with compounds 41-60 were labeled with Yellow-DCG-04. After one hour labeling the samples were quenched by addition of SDS sample buffer. The yellow, blue, and green samples were mixed and a small portion was analyzed by SDS-PAGE and laser scanning on an ABI 377 5 DNA sequencer. This image shows a typical gel image generated from scanning of the gel as well as the process by which labeled bands can be quantitated (panel to left). Small molecules can be analyzed for their potency and selectivity for targets in the rat liver proteasome using this method. Note that each color data can be separately extracted due to non-overlapping emission spectra of the chosen fluorophores. This approach therefore 10 allows analysis of up to 80 samples in a single gel using four color labels.

[0233] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, 15 and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

CLAIMS

What is claimed is:

1. A system for identifying papain cysteine hydrolases comprising the use in combination of at least two compounds of the formula:



where:

A is at least 15 Dal and not more than about 2 kDal and is a ligand; L¹ and L² may be the same or different and are a bond or a chain of from 1 to 40, atoms;

10 Hy is a hydrophobic group that specifically binds in the papain cysteine protease pocket.; and

E is an epoxide that covalently bonds to the active site of the papain cysteine hydrolase.

2. A system according to claim 1, wherein A is a detectable ligand.

15 3. A system according to claim 2, wherein said detectable ligand is a fluorescer.

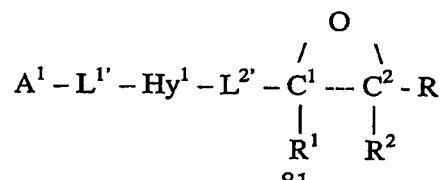
4. A system according to claim 1, wherein A is a ligand that binds to a naturally occurring receptor.

20 5. A system according to claim 1, wherein Hy is an aliphatic, aromatic or alicyclic side chain bonded to a carbon chain linking an amino group to a carboxy group.

6. A system according to claim 1, wherein each of said compounds has a radioactive label.

7. A compound of the formula:

25



wherein:

A¹ is a moiety that provides a detectable signal or a ligand;
L¹ and L² are the same or different and are a bond or an aliphatic chain of from 1 to 8 carbon atoms joined to A¹ or the epoxide C¹ annular carbon atom and
5 Hy¹ through the same or different functional groups;

Hy¹ is a neutral hydrophobic amino acid having a total of at least 4 and not more than about 20 carbon atoms, having a side chain of at least about 2 carbon atoms and lacking a quaternary carbon atom;

the R groups are the same or different there being not more than two
10 of the R groups other than hydrogen, where the total number of carbon atoms for all of the R groups is from 0 to 8.

8. A compound according to claim 7, wherein A¹ is a fluorescer.

9. A compound according to claim 7, wherein said epoxide is a single stereoisomer.

15 10. A compound according to claim 7, wherein A¹ is a ligand.

11. A compound according to claim 7, wherein Hy¹ comprises a carbocyclic side chain.

12. A compound according to claim 7, wherein Hy¹ comprises an acyclic aliphatic side chain.

20 13. A cell comprising a papain cysteine hydrolase bonded to an hydroxyethylene group of an epoxide compound as a result of a reaction between said papain cysteine hydrolase and an annular carbon atom of said epoxide compound, said epoxide compound of the formula:



25 wherein:

A is at least 15 Dal and not more than about 2 kDal and is a ligand;
L¹ and L² may be the same or different and are a bond or a chain of from 1 to 40, atoms;

Hy is a hydrophobic group that specifically binds in the papain cysteine protease pocket; and

E is an epoxide.

14. A cell according to claim 13, wherein said ligand is a fluorescer.

5 15. A cell according to claim 13, wherein said epoxide compound comprises a radioactive label.

16. A method for determining the presence of at least one active papain cysteine hydrolase target in a sample, said method comprising:

combining said sample with at least one compound of the formula:

10 A - L¹ - Hy - L² - E

where:

A is at least 15 Dal and not more than about 2 kDal and is a ligand;

15 L¹ and L² may be the same or different and are a bond or a chain of from 1 to 40, atoms;

Hy is a hydrophobic group that specifically binds in said papain cysteine protease pocket; and

20 E is an epoxide that covalently bonds to the active site of the papain cysteine hydrolase under conditions wherein said papain cysteine hydrolase target reacts with said epoxide to form a covalently linked conjugate; and

determining the presence of said papain cysteine hydrolase by means of said ligand.

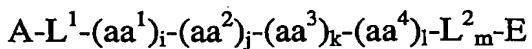
17. A method according to claim 16, wherein said papain cysteine hydrolase and said compound are present in a cell.

25 18. A method according to claim 16, wherein said compound comprises a radioactive label or said ligand is a fluorescer label and said determining comprises detecting said label.

19. A method according to claim 16, including the additional step of sequestering said covalently linked conjugate by means of said ligand.

20. A method according to claim 16, wherein a plurality of compounds are combined, each of said compounds having a different profile of binding to papain cysteine hydrolases.

21. A probe for monitoring or identifying cysteine hydrolase activity, 5 said probe comprising a compound having the formula:



wherein A is a ligand or a detectable label;

L^1 is a linker;

L^2 , when present, is a linker;

10 aa^1 , aa^2 , aa^3 , and aa^4 , when present, are independently selected amino acids;

i, j, k, l, and m are independently 0 or 1;

E is an electrophile; and

at least two of aa^1 , aa^2 , aa^3 , and aa^4 are present.

15

22. The probe of claim 21, wherein A is a detectable label.

23. The probe of claim 21, wherein A is a fluorescent label.

24. The probe of claim 21, wherein at least one of aa^1 , aa^2 , aa^3 , and aa^4 is labeled with a detectable label.

20 25. The probe of claim 24, wherein A is a ligand.

26. The probe of claim 24, wherein said detectable label is a radioactive label selected from the group consisting of 3H , ^{125}I , ^{35}S , ^{14}C , and ^{32}P .

27. The probe of claim 21, wherein aa^1 , aa^2 , aa^3 , and aa^4 , when present, are independently selected from the group consisting of alanine, valine, leucine, 25 isoleucine, proline, phenylalanine, tryptophan, methionine, aspartic acid, glutamic acid, lysine, arginine, histidine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, and norleucine.

28. The probe of claim 21, wherein said electrophile is selected from the group consisting of a diazomethyl ketone, a fluoromethyl ketones, an acyloxymethyl ketone, a chloromethyl ketone, an o-acylhydroxylamine, a vinyl sulfone, an epoxysuccinic derivative, and an epoxide.

5 29. The probe of claim 21, wherein A is an affinity tag.

30. The probe of claim 29, wherein A is an affinity tag is selected from the group consisting of a biotin, an avidin, a streptavidin, an antibody, and an epitope tag.

31. The probe of claim 30, wherein A is an epitope tag selected from the group consisting of a polyhistidine, a polyarginine, a Flag-tag, an HA-tag, a myc-tag, 10 and a DYKDDDDK epitope.

32. The probe of claim 21, wherein L¹ and L², when present, are independently selected from the group consisting of a straight chain carbon linker, a branched-chain carbon linkers, a cleavable linker, and a heterocyclic carbon linker.

33. The probe of claim 32, wherein L¹ and L², when present, are 15 independently selected straight chain C₁ to C₂₀ carbon linkers.

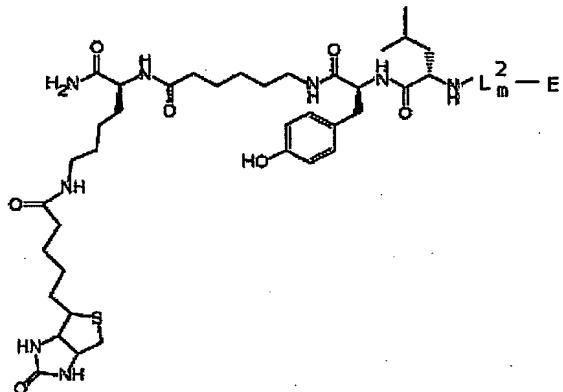
34. The probe of claim 32, wherein L¹ is a hexanoic acid linker.

35. The probe of claim 32, wherein L¹ is a photolabile cleavable linker or an oxidizable cleavable linker.

36. The probe of claim 21, wherein:
20 i and j are zero; and
k and l are 1.

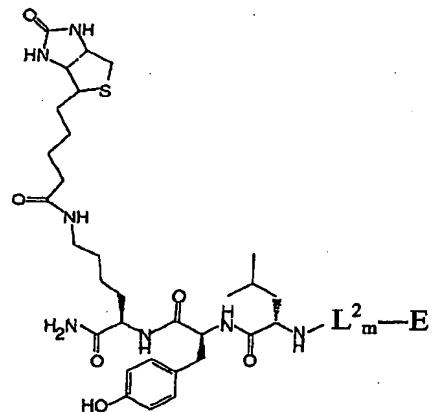
37. The probe of claim 36, wherein:
aa³ is (tyrosine); and
aa⁴ is selected from the group consisting of alanine, valine, leucine, 25 isoleucine, proline, phenylalanine, tryptophan, methionine, aspartic acid, glutamic acid, lysine, arginine, histidine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, and norleucine.

38. The probe of claim 37, wherein L^1 is an amino hexanoic acid spacer and A is a biotin.
39. The probe of claim 38, wherein said E is an epoxide.
40. The probe of claim 21, wherein said probe comprises the formula:

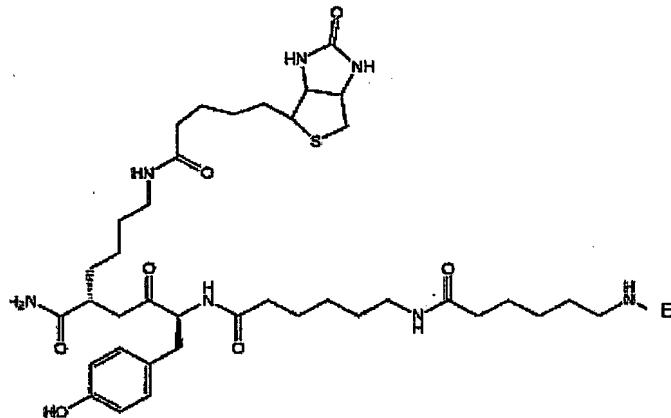


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41. The probe of claim 21, wherein said probe comprises the formula:



42. The probe of claim 21, wherein said probe comprises the formula:



43. The probe of claim 21, wherein said probe comprises the formula of BODIPY558/568-DCG-04.

44. The probe of claim 21, wherein said probe comprises the formula of
5 BODIPY493/503-DCG-04. -

45. The probe of claim 21, wherein said probe comprises the formula of
, BODIPY530/550-DCG-04.

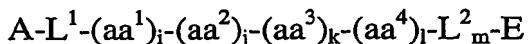
46. The probe of claim 21, wherein said probe comprises the formula of BODIPY588/616-DCG-04.

10 47. The probe of claim 21, wherein said compound is selected from the group consisting of DCG-01, DCG-04, and DCG-03.

48. The probe of claim 21, wherein said probe is attached to a solid support.

49. The probe of claim 48, wherein said probe is attached to a solid support A where A is a ligand.

50. A probe library for monitoring or identifying cysteine protease activity, said probe library comprising a plurality of members each member of said plurality of members comprising a compound having the formula:



wherein A is a ligand or a detectable label;
 L^1 is a linker;
 L^2 , when present, is a linker;
 aa^1 , aa^2 , aa^3 , and aa^4 , when present, are independently selected

5 amino acids;

i, j, k, l, and m are independently 0 or 1;
E is an electrophile; and
at least two of aa^1 , aa^2 , aa^3 , and aa^4 are present.

51. The probe library of claim 50, wherein A is a detectable label.
- 10 52. The probe library of claim 51, wherein A is a fluorescent label.
53. The probe library of claim 50, wherein at least one of aa^1 , aa^2 , aa^3 , and aa^4 is labeled with a detectable label.
54. The probe library of claim 53, wherein A is a ligand.
- 15 55. The probe library of claim 53, wherein said detectable label is a radioactive label selected from the group consisting of 3H , ^{125}I , ^{35}S , ^{14}C , and ^{32}P .
56. The probe library of claim 50, wherein aa^1 , aa^2 , aa^3 , and aa^4 , when present, are independently selected from the group consisting of alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, aspartic acid, glutamic acid, lysine, arginine, histidine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, and norleucine.
- 20 57. The probe library of claim 50, wherein said electrophile is selected from the group consisting of a diazomethyl ketone, a fluoromethyl ketone, an acyloxymethyl ketone, a chloromethyl ketone, an o-acylhydroxylamine, a vinyl sulfone, an epoxysuccinic derivative, and an epoxide.
- 25 58. The probe library of claim 50, wherein A is an affinity tag.

59. The probe library of claim 58, wherein A is an affinity tag selected from the group consisting of a biotin, an avidin, a streptavidin, an antibody, and an epitope tag.

60. The probe library of claim 59, wherein A is an affinity tag that is an epitope tag selected from the group consisting of a polyhistidine, a polyarginine, a Flag-tag, an HA-tag, a myc-tag, and a DYKDDDDK epitope.

61. The probe library of claim 50, wherein L¹ and L², when present, are independently selected from the group consisting of a straight chain carbon linker, a branched-chain carbon linkers, and a heterocyclic carbon linker.

62. The probe library of claim 61, wherein L¹ and L², when present, are independently selected straight chain C₁ to C₂₀ carbon linkers.

63. The probe library of claim 61, wherein L¹ is a hexanoic acid linker.

64. The probe library of claim 50, wherein:

i and j are zero; and

15 k and l are 1.

65. The probe library of claim 64, wherein:

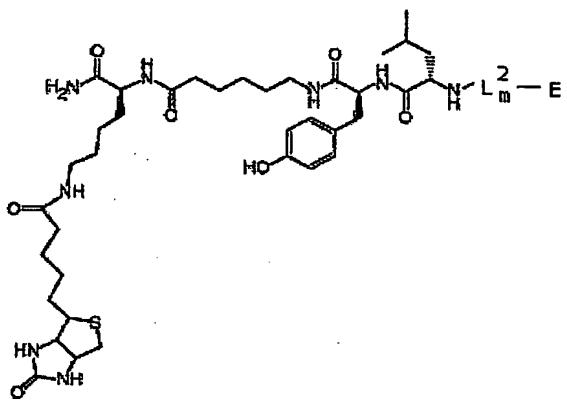
aa³ is (tyrosine) and

20 aa⁴ is selected from the group consisting of alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, aspartic acid, glutamic acid, lysine, arginine, histidine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, and norleucine.

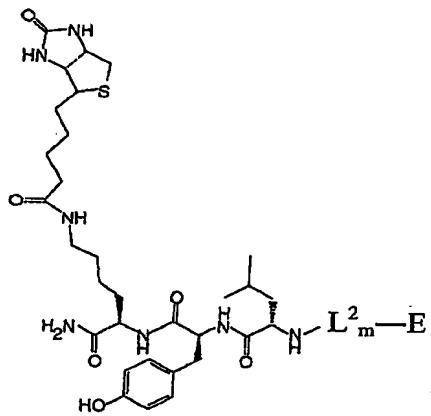
66. The probe library of claim 65, wherein L¹ is an amino hexanoic acid spacer and A is a biotin.

67. The probe library of claim 66, wherein E is an epoxide.

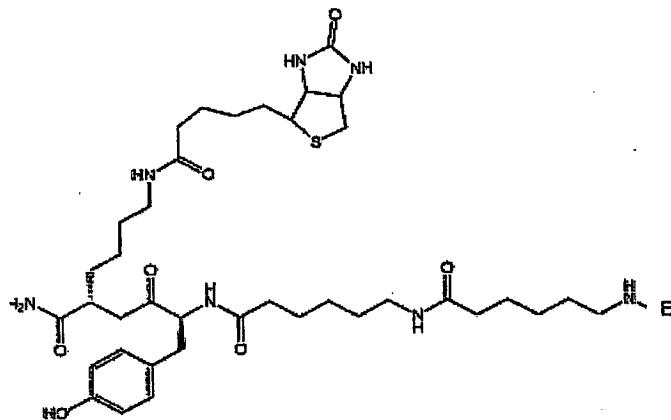
25 68. The probe of claim 50, wherein said compound has the formula:



69. The probe of claim 50, wherein said compound has the formula:



70. The probe of claim 50, wherein said compound has the formula:



71. The probe library of claim 50, wherein said probe comprises the formula of BODIPY558/568-DCG-04.

72. The probe library of claim 50, wherein said probe comprises the formula of BODIPY493/503-DCG-04.

73. The probe library of claim 50, wherein said probe comprises the formula of BODIPY530/550-DCG-04.

74. The probe library of claim 50, wherein said probe comprises the formula of BODIPY588/616-DCG-04.

75. The probe library of claim 50, wherein said library comprises at least 10 different members.

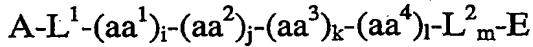
76. The probe library of claim 50, wherein said library comprises at least 20 different members.

77. The probe library of claim 50, wherein said compounds are attached to a solid support.

78. The probe library of claim 77, wherein the compounds are attached to a solid support through said affinity tag.

79. A method of identifying or determining activity of a cysteine hydrolase, said method comprising:

i) providing a biological sample;
ii) contacting said biological sample with a compound having the formula:



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wherein A is a ligand or a detectable label;

L^1 is a linker;

L^2 , when present, is a linker;

aa^1 , aa^2 , aa^3 , and aa^4 , when present, are independently

selected amino acids;

10

i, j, k, l, and m are independently 0 or 1;

E is an electrophile; and

at least two of aa^1 , aa^2 , aa^3 , and aa^4 are present; and

iii) detecting specific binding of said compound to a component of said biological sample whereby said detecting identifies or quantifies a cysteine hydrolase.

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80. The method of claim 79, wherein A is a detectable label.

81. The method of claim 80, wherein A is a fluorescent label.

82. The method of claim 79, wherein at least one of aa^1 , aa^2 , aa^3 , and aa^4 is labeled with a detectable label.

83. The method of claim 82, wherein said A is a ligand.

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84. The method of claim 82, wherein said detectable label is a radioactive label selected from the group consisting of 3H , ^{125}I , ^{35}S , ^{14}C , and ^{32}P .

85. The method of claim 79, wherein said biological sample comprises a crude cellular extract.

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86. The method of claim 79, wherein said biological sample comprises a purified protein.

87. The method of claim 84, wherein said detecting comprises detecting direct labeling of said component by detecting the label on said compound.

88. The method of claim 79, wherein said method further comprises contacting said biological sample with a known inhibitor of a cysteine hydrolase and determining the amount of binding of said compound competed by said inhibitor of a cysteine hydrolase.

5 89. The method of claim 79, wherein said detecting comprises contacting a control comprising a denatured biological sample with said compound and detecting the differences between the binding of said compound to said sample and said compound to said control.

10 90. The method of claim 79, wherein said detecting further comprises isolating a component specifically bound by said compound by contacting said compound with a ligand that binds to said affinity tag.

91. The method of claim 90, wherein said affinity tag is a biotin and said ligand is a streptavidin or a modified streptavidin.

15 92. The method of claim 90, wherein said affinity tag is a poly-His tag and said ligand is a Ni-NTA.

93. The method of claim 90, wherein said method further comprises digesting the component bound by said compound.

94. The method of claim 90, wherein said method further comprises performing an amino acid analysis of the component bound by said compound.

20 95. The method of claim 90, wherein said method further comprises performing mass spectroscopy of the component bound by said compound.

96. The method of claim 79, wherein said biological sample is a crude cellular extract and the binding profile of said probe is compared to binding profiles stored in a specificity fingerprint database to identify a protease in said extract.

25 97. The method of claim 79, wherein said detecting comprises comparing a binding profile of said probe to one or more components of said sample to the

binding profile of the members of said library to one or more components of a second sample.

5 98. The method of claim 79, wherein said detecting comprises comparing specific binding of the compound to a component of the biological sample with the binding of the compound to one or more components of a sample from a different cell or tissue.

99. The method of claim 98, wherein said biological sample is a sample from a pathological or diseased cell or tissue and said different cell or tissue is a healthy cell or tissue.

10 100. The method of claim 79, wherein said compound is a member of a library of cysteine hydrolase probes comprising a plurality of different cysteine hydrolase probes and said contacting comprises contacting said biological sample with said library.

15 101. The method of claim 100, wherein said biological sample is a purified protease and binding of each member of said library to said protease is recorded to produce a specificity fingerprint for said protease.

102. The method of claim 101, wherein said specificity fingerprint is entered into a database of specificity fingerprints for various proteases.

20 103. The method of claim 100, wherein said biological sample is a crude cellular extract and the binding profile of the members of said library is compared to binding profiles stored in a specificity fingerprint database to identify a protease in said extract.

25 104. The method of claim 100, wherein said detecting comprises comparing a binding profile of the members of said library to one or more components of said sample to the binding profile of the members of said library to one or more components of a second sample.

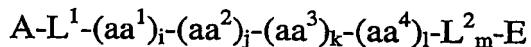
105. The method of claim 100, wherein said detecting comprises comparing a binding profile of the members of said library to one or more components of

the biological sample with the a binding profile of the members of said library to one or more components of a sample from a different cell or tissue.

106. The method of claim 105, wherein said biological sample is a sample from a pathological or diseased cell or tissue and said different cell or tissue is a
5 healthy cell or tissue.

107. A method of identifying an agent that modulates activity of a cysteine hydrolase, said method comprising:

- i) providing a biological sample;
- ii) contacting said biological sample with a compound having the
10 formula:



wherein A is a ligand or a detectable label;

L¹ is a linker;

L², when present, is a linker;

15 aa¹, aa², aa³, and aa⁴, when present, are independently selected amino acids;

i, j, k, l, and m are independently 0 or 1;

E is an electrophile; and

at least two of aa¹, aa², aa³, and aa⁴ are present;

- iii) contacting said biological sample with a test agent; and
- iv) detecting specific binding of said compound to a component of said biological sample whereby a difference in the binding of said compound to a component of said biological sample as compared to the binding of said compound to a component of said biological sample where said test agent is absent or present at a lower concentration indicates that said test agent modulates activity of said cysteine hydrolase.

20 108. The method of claim 107, wherein A is a detectable label.

109. The method of claim 108, wherein A is a fluorescent label.

110. The method of claim 107, wherein at least one of aa¹, aa², aa³, and aa⁴ is labeled with a detectable label.

111. The method of claim 107, wherein A is a ligand.
112. The method of claim 107, wherein said detectable label is a radioactive label selected from the group consisting of ^3H , ^{125}I , ^{35}S , ^{14}C , and ^{32}P .
113. The method of claim 107, wherein said biological sample comprises
5 a crude cellular extract.
114. The method of claim 107, wherein said biological sample comprises a purified protein.
115. The method of claim 114, wherein said detecting comprises detecting direct labeling of said component by detecting the label on said compound.
116. The method of claim 107, wherein said detecting comprises
10 contacting a control comprising a denatured biological sample with said compound and detecting the differences between the binding of said compound to said sample and said compound to said control.
117. The method of claim 107, wherein said compound is a member of a
15 library of said compounds comprising a plurality of different compounds and said contacting comprises contacting said biological sample with said library.
118. The method of claim 107, wherein said detecting further comprises isolating a component specifically bound by said compound by contacting said compound with a ligand that binds to said affinity tag.
119. The method of claim 118, wherein said affinity tag is a biotin and
20 said ligand is a streptavidin or a modified streptavidin.
120. The method of claim 107, wherein said biological sample is a purified protease and binding of each member of said library to said protease is recorded to produce a modulation fingerprint for said test agent.
121. The method of claim 120, wherein said specificity fingerprint is
25 entered into a database of modulation fingerprints for various agents.

122. The method of claim 107, wherein said biological sample is a crude cellular extract and the pattern of binding of the members of said library is compared to patterns of binding stored in a specificity fingerprint database to classify a test agent's mode of activity.

5 123. The method of claim 107, wherein said detecting comprises comparing specific binding of the compound to a component of the biological sample with the binding of the compound to a component of a sample from a different cell or tissue.

124. A method of synthesizing an inhibitor of a cysteine protease said method comprising:

10 synthesizing an oligopeptide in a solid phase peptide synthesis procedure;

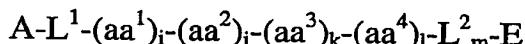
coupling a (2s,3s)-oxirane-2,3-dicarboxylate to said oligopeptide; and

15 cleaving said peptide from said solid support to produce an oligopeptide bearing an epoxide.

125. The method of claim 124, wherein said oligopeptide is a dipeptide.

126. The method of claim 124, wherein said cleaving uses trifluoroacetic acid (TFA).

127. A kit for monitoring or identifying cysteine hydrolase activity, said 20 kit comprising a container containing a compound having the formula:



wherein A is a ligand or a detectable label;

L^1 is a linker;

L^2 , when present, is a linker;

25 aa^1 , aa^2 , aa^3 , and aa^4 , when present, are independently selected amino acids;

i , j , k , l , and m are independently 0 or 1;

E is an electrophile; and

at least two of aa^1 , aa^2 , aa^3 , and aa^4 are present.

128. The kit of claim 127, wherein A is a detectable label.
129. The kit of claim 128, wherein A is a fluorescent label.
130. The kit of claim 127, wherein at least one of aa¹, aa², aa³, and aa⁴ is labeled with a detectable label.
5 131. The kit of claim 130, wherein A is a ligand.
132. The kit of claim 130, wherein said detectable label is a radioactive label selected from the group consisting of ³H, ¹²⁵I, ³⁵S, ¹⁴C, and ³²P.
133. The kit of claim 127, further comprising a known inhibitor of a cysteine hydrolase.
10 134. The kit of claim 127, wherein said compound is a member of a library of cysteine hydrolase probes comprising a plurality of different cysteine hydrolase probes and said kit comprises said library.
15 135. The kit of claim 127, wherein said kit further comprises instructional materials providing protocols for using a cysteine hydrolase probe to monitor or identify cysteine hydrolase activity or to isolate a cysteine hydrolase.

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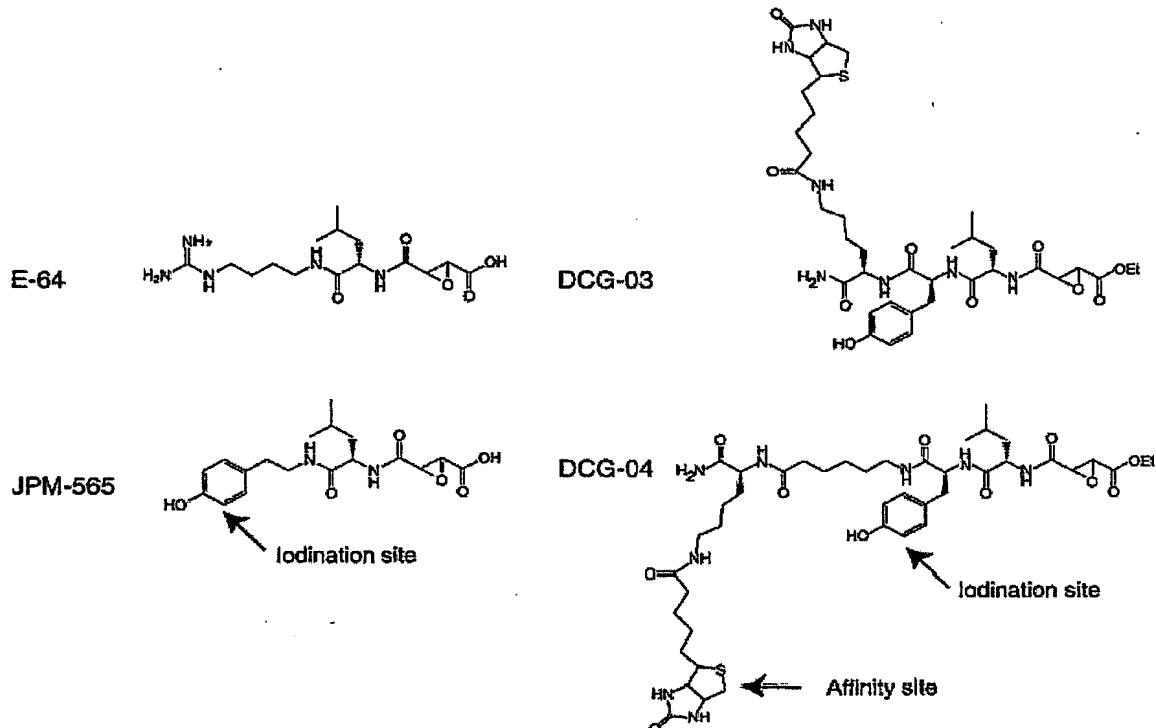


Fig. 1

Fig. 2A

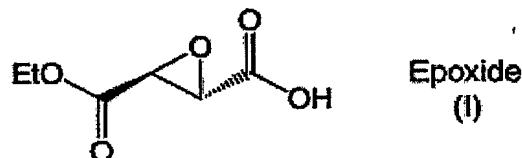
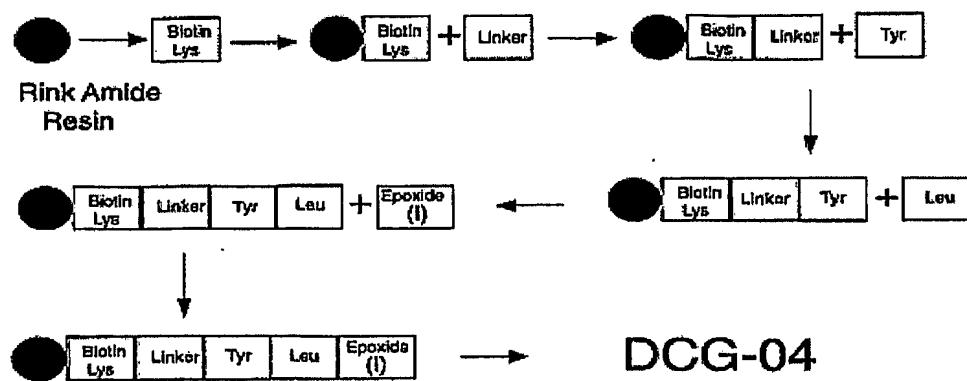


Fig. 2B



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Fig. 3A
Fig. 3A_{7,4}
Fig. 3A_{5,5}

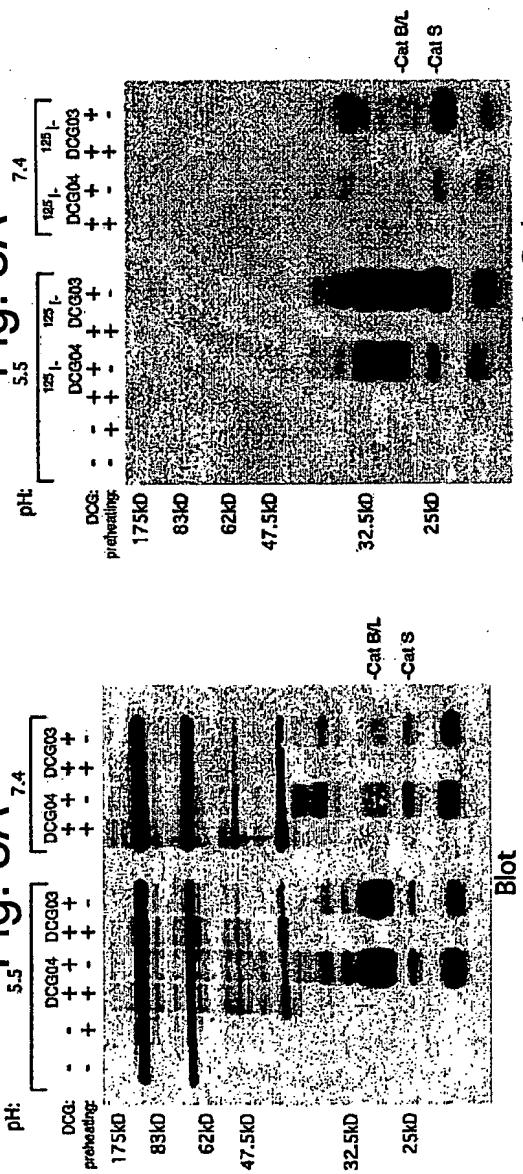
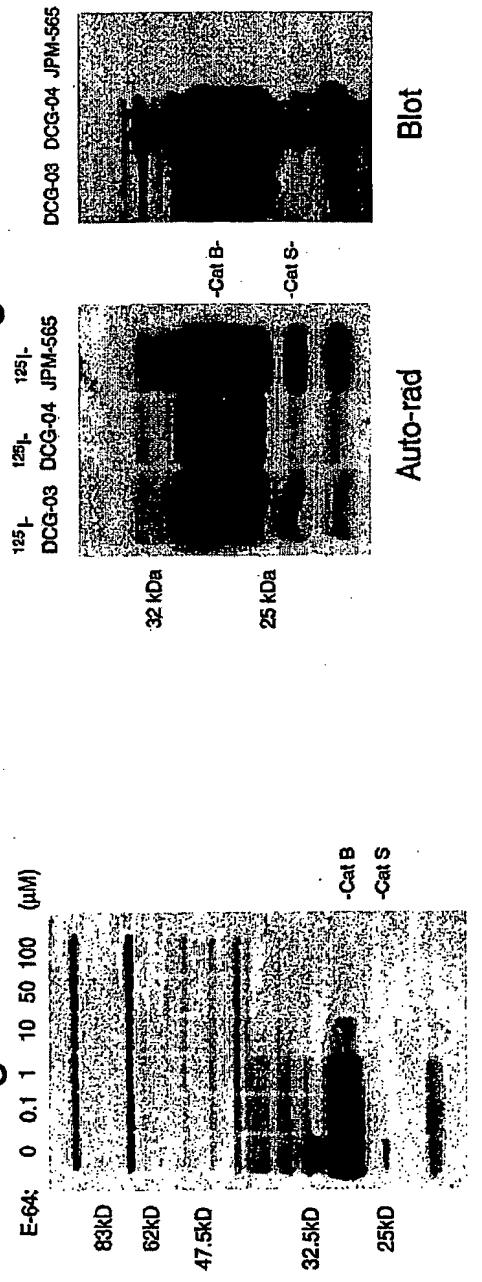


Fig. 4B



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Fig. 5A

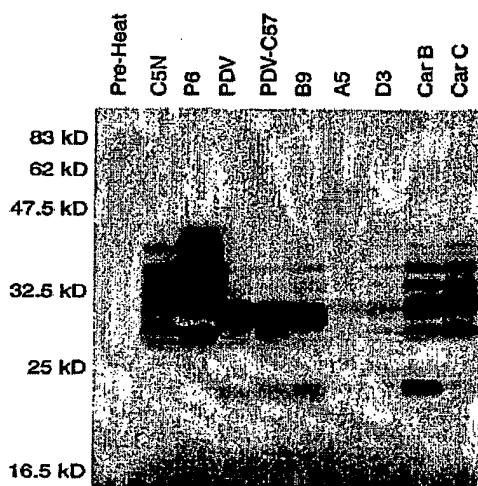


Fig. 5B

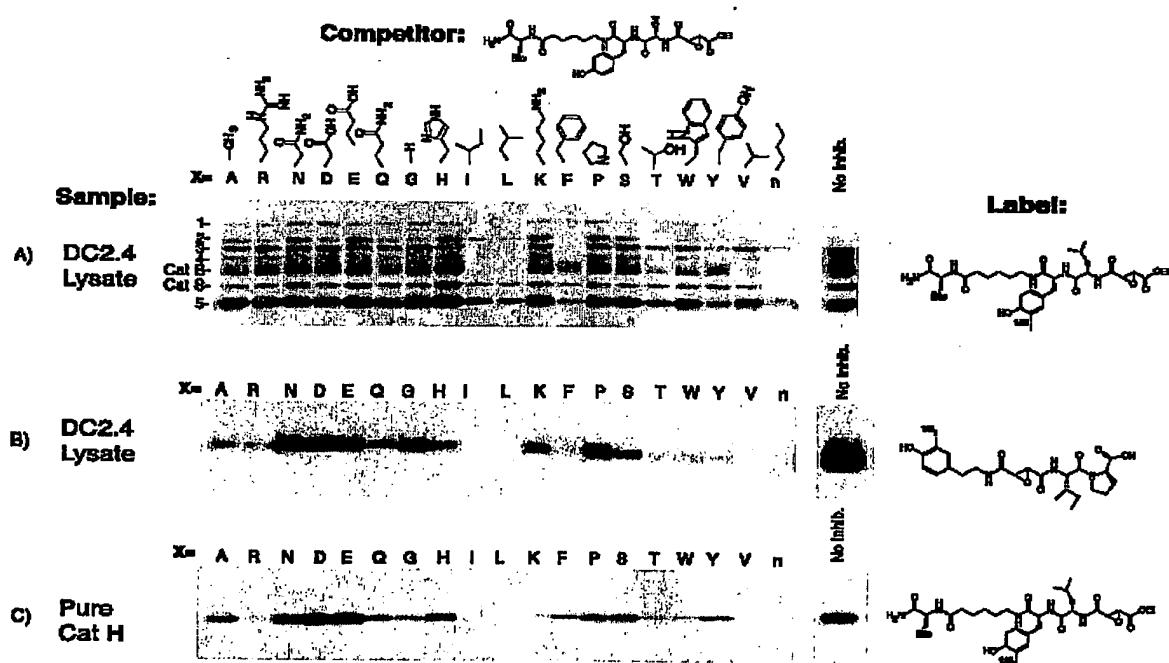
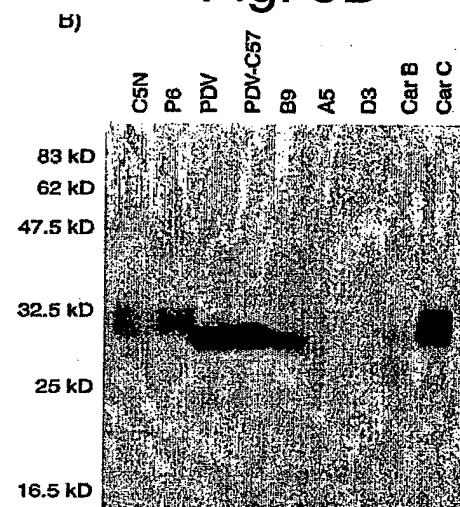


Fig. 6

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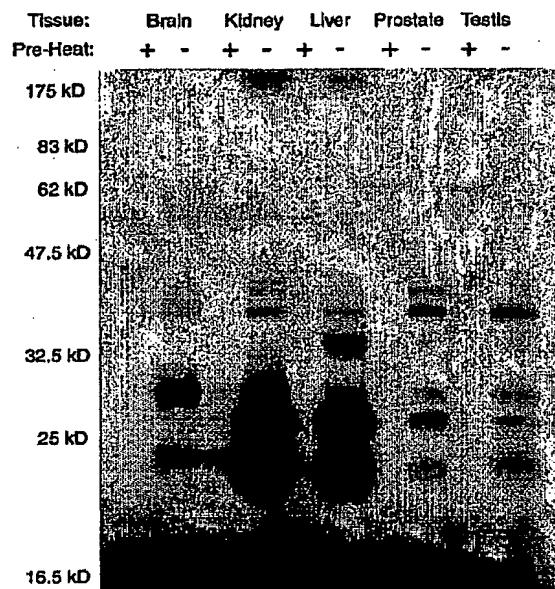


Fig. 7

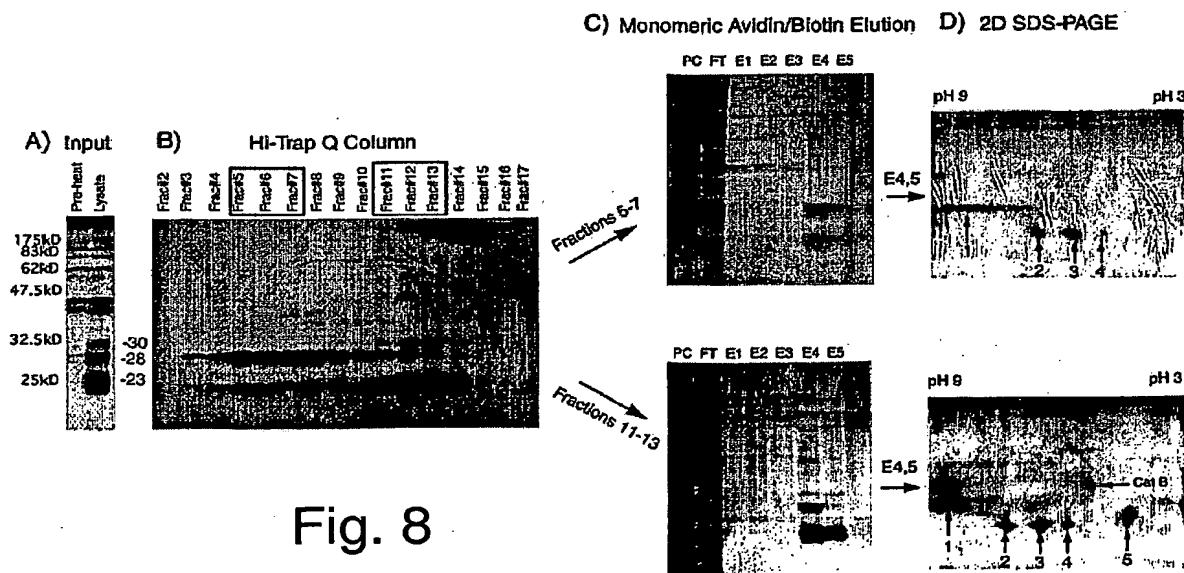


Fig. 8

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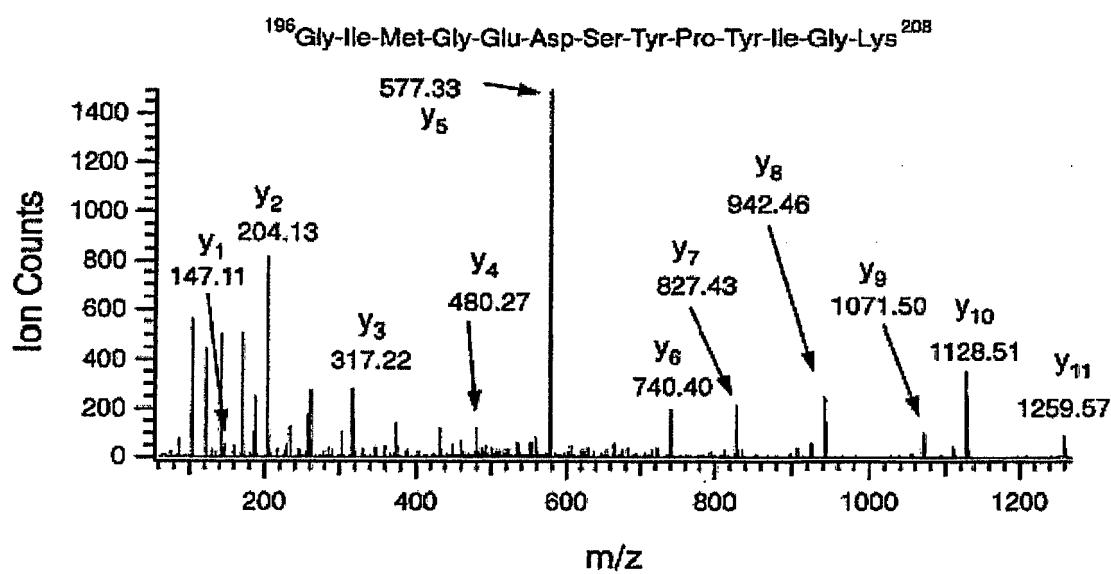


Fig. 9

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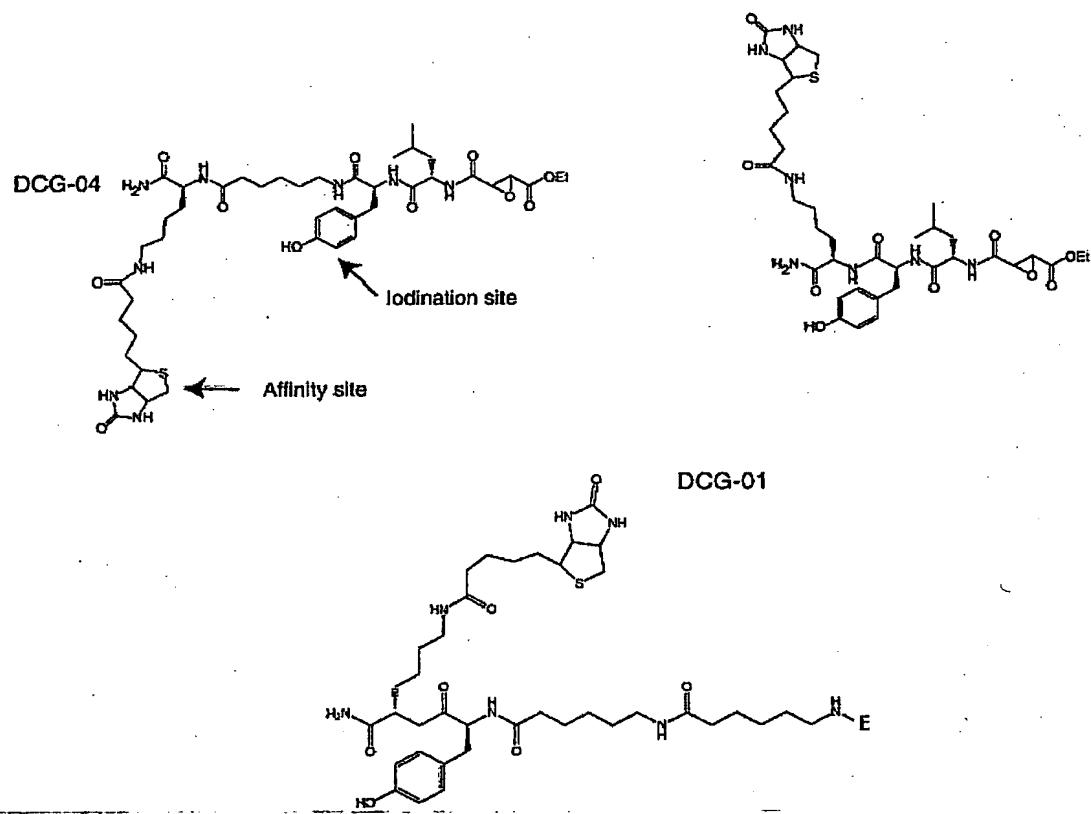


Fig. 10

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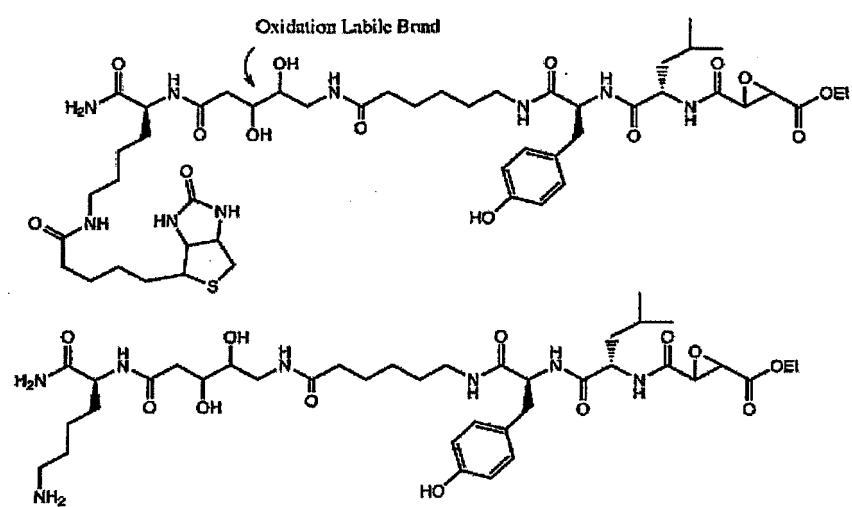


Fig. 11

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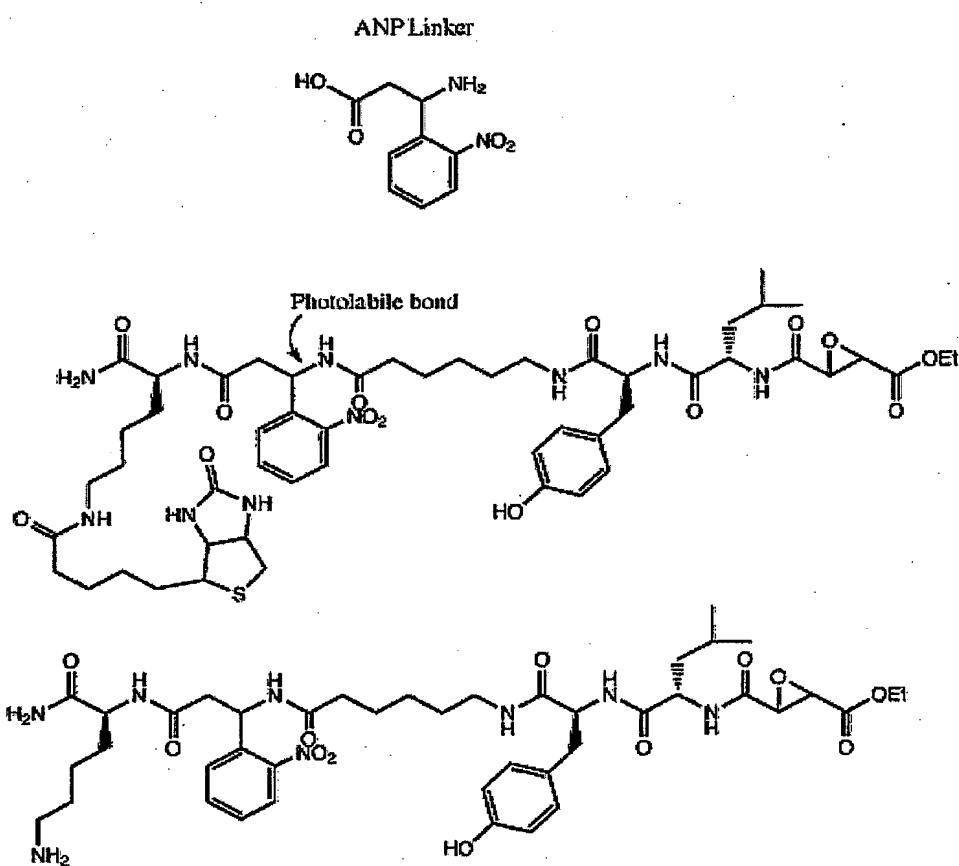
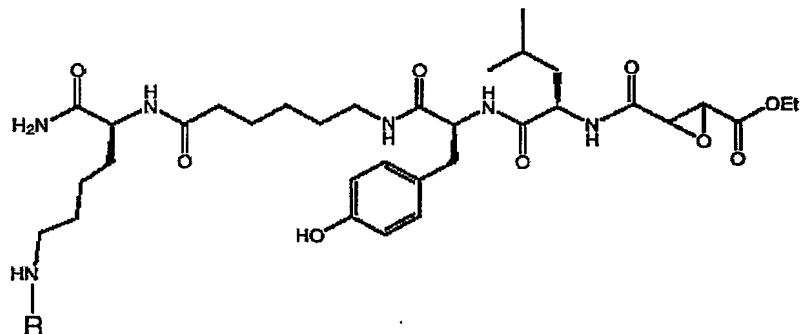
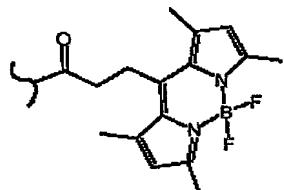
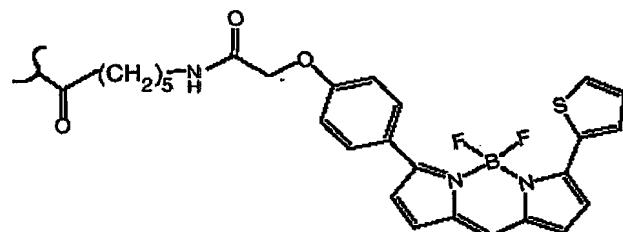


Fig. 12

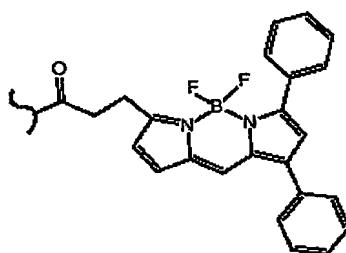
9/20

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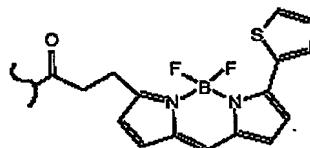
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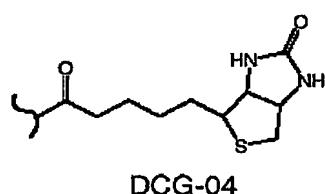


Fig. 13

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Cathepsin: C S F H K V B L

30kDa-

25kDa-

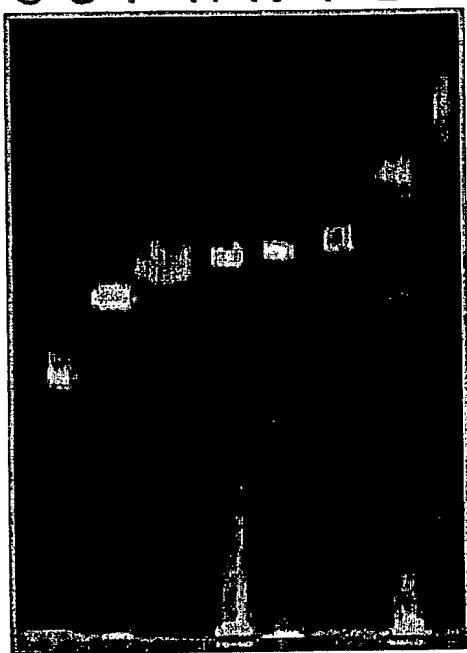


Fig. 14A

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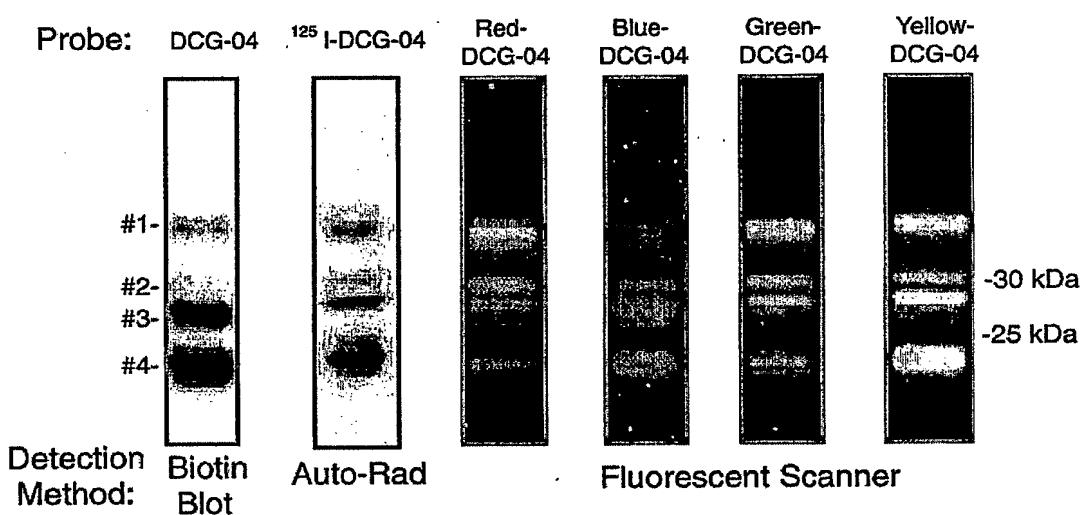


Fig. 14B

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Green-DCG-04 Labeled

SDS-
Page

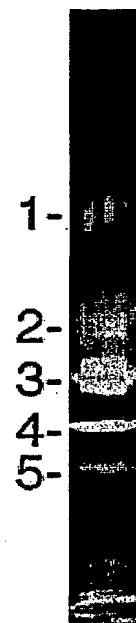
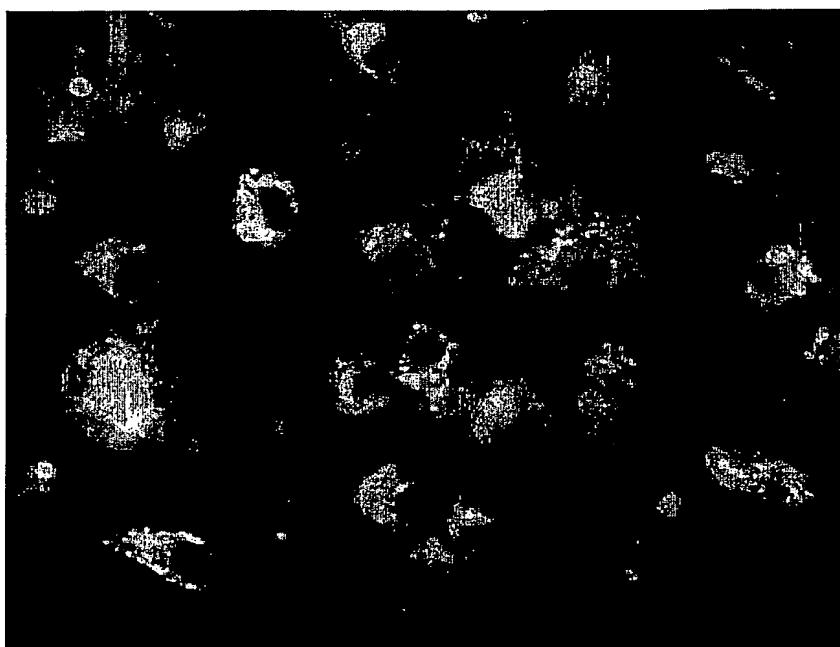


Fig. 15A

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E64 Pre-Treated
Green-DCG-04 Labeled

SDS-
Page



Fig. 15B

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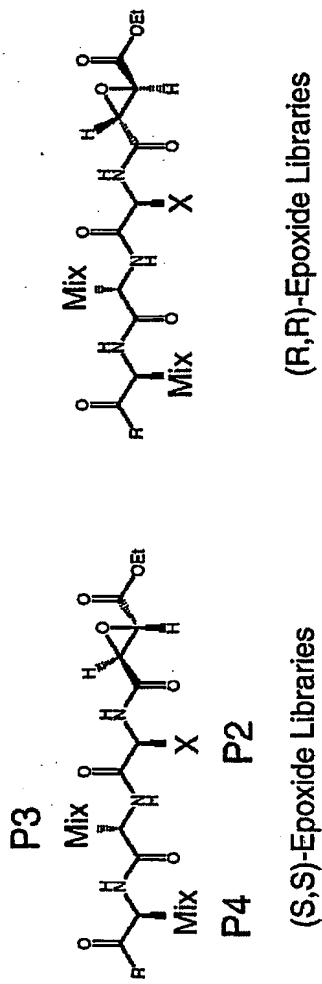


Fig. 16A

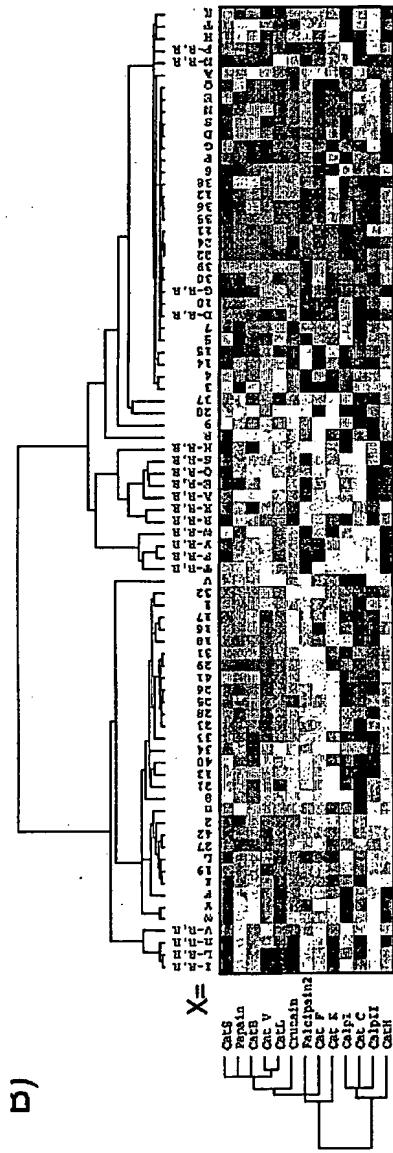
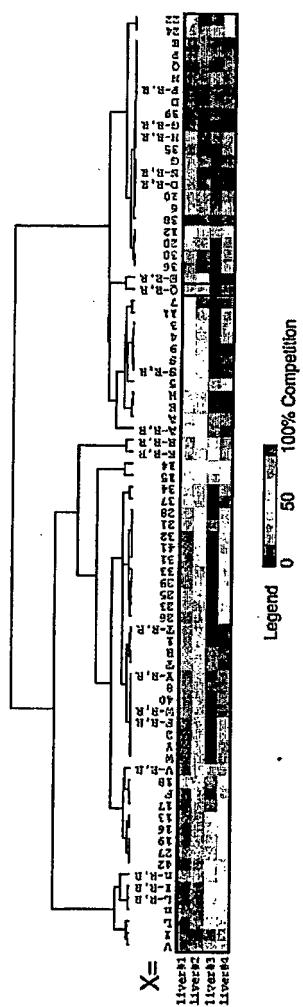


Fig. 16B

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SUBSTITUTE SHEET (RULE 26)

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acidic

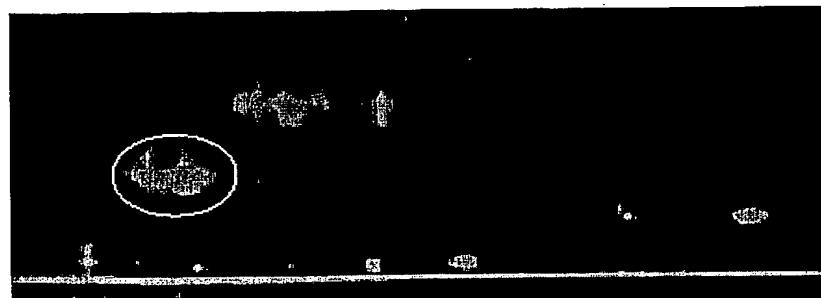
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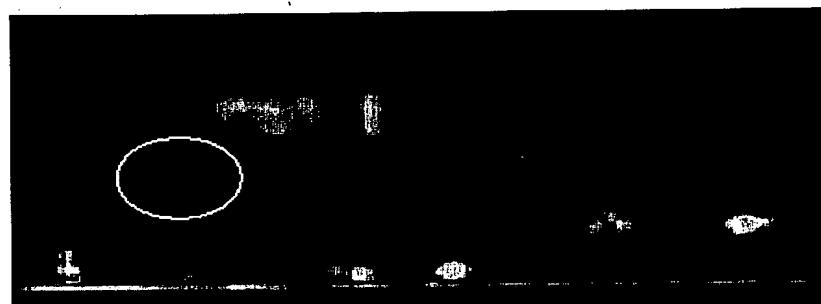
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#3-

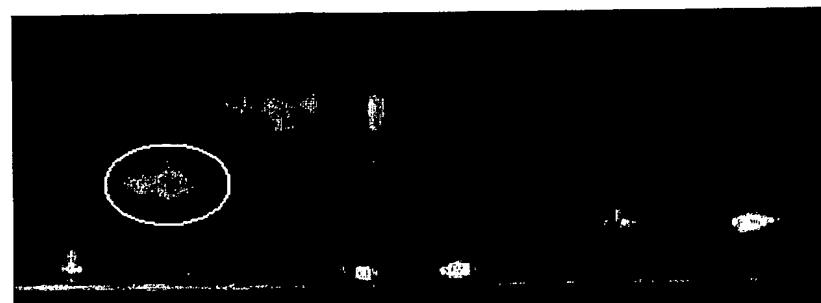
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Untreated-Red Channel



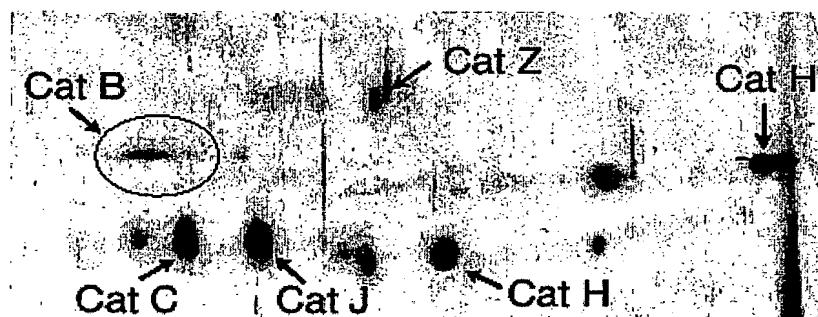
Treated-Blue Channel



Both Channels

Fig. 17A

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Affinity Purification

Fig. 17B

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A)

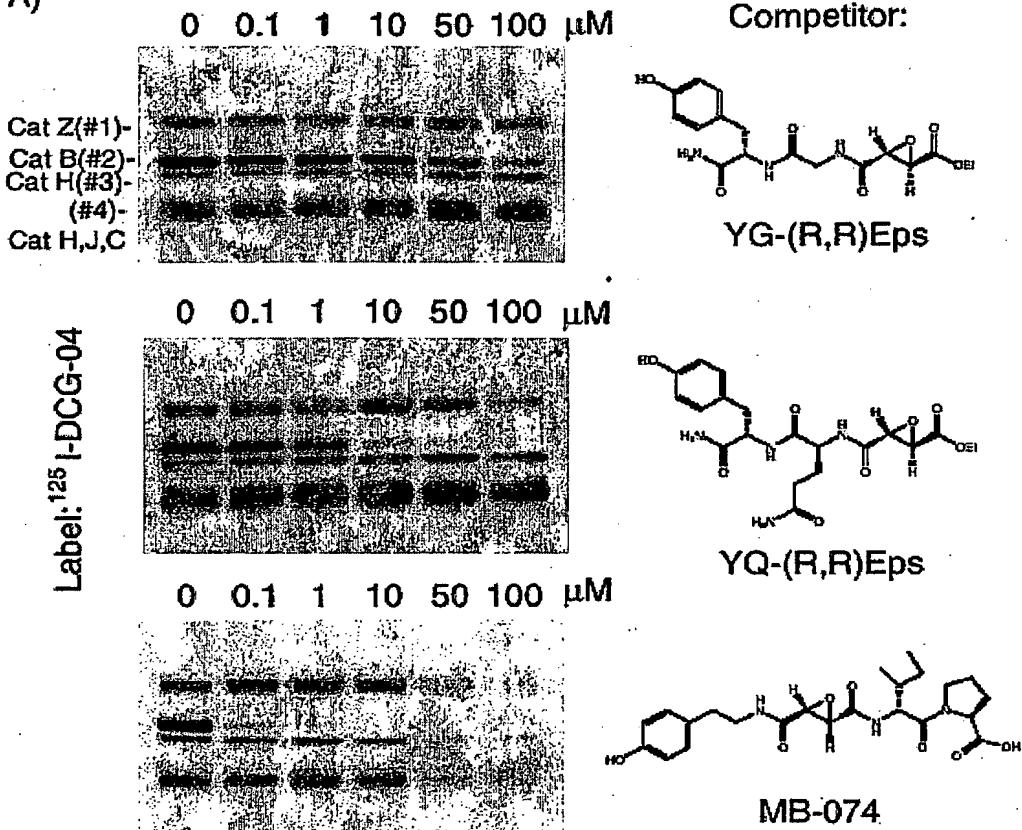


Fig. 18A

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B)

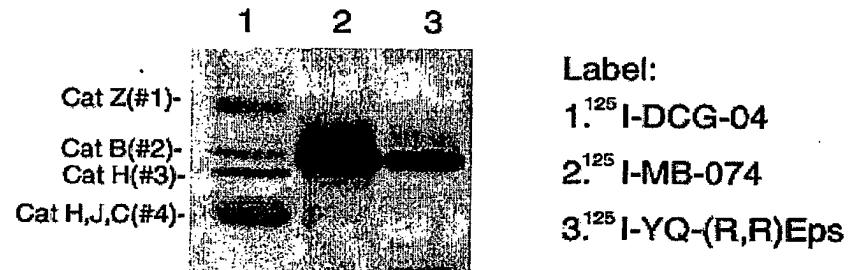


Fig. 18B

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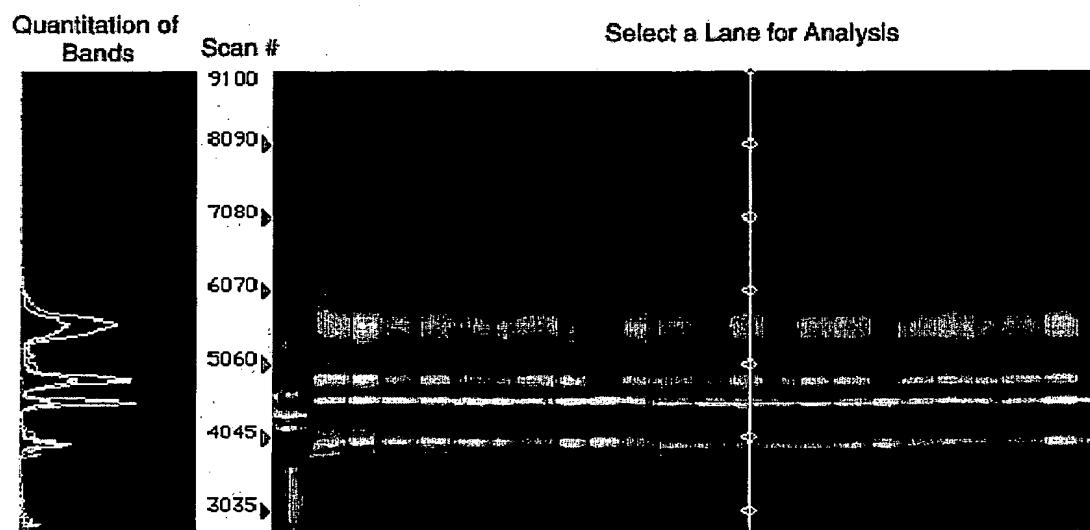


Fig. 19

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<140> unassigned

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<151> 2000-11-10

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<151> 2000-05-01

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1 5 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/49480

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 485

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---	US 5,935,959 A (INOUE et al) 10 August 1999, see entire document, especially columns 2-3 and Tables.	1, 2, 4, 5, 7, 9, 10, 12 ---
Y		3, 6, 8, 11, 21-78
X ---	YAMADA et al. Cysteine Protease Inhibitors Produced by the Industrial Koji Mold, Aspergillus oryzae O-1018. Biosci. Biotechnol. Biochem. 1998, Vol. 62, No. 5, pages 907-914, see Abstract, Figure 3 and Table 5.	1, 2, 4, 5, 7, 9, 10, 12 ---
Y		3, 6, 8, 11, 21-781, 2,
X ---	YAGINUMA et al. Isolation and Characterization of New Thiol Protease Inhibitors Estatins A and B. J. Antibiotics. September 1989, Vol. 42, No. 9, pages 1362-1369, see Abstract, Figure 1 and Table 4.	1, 2, 4, 5, 7, 9, 10, 12 ---
Y		3, 6, 8, 11, 21-78

Further documents are listed in the continuation of Box C. See patent family annex.

•	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
08 MAY 2002

Date of mailing of the international search report

31 MAY 2002

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231
Facsimile No. (703) 305-3290

Authorized officer
MAURIE E. GARCIA
Telephone No. (703) 305-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/49480

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/32954 A1 (TAKEDA CHEMICAL INDUSTRIES, LTD.) 07 December 1995, see entire document, especially Abstract and pages 29-30.	1, 2, 4, 5, 7, 9, 10, 12
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Y		3, 6, 8, 11, 21-78
X	US 5,776,718 A (PALMER et al) 07 July 1998, see entire document, especially columns 2-5, Figures and Table 2 (column 45).	21, 22, 27, 32, 33, 36, 50, 51, 56, 61, 62, 64, 75, 76
---		---
Y		1-12, 23-26, 28-31, 34, 35, 37-49, 52-55, 57-60, 63, 65-74, 77, 78
X	LEE et al. A Substrate Combinatorial Array for Caspases. Bioorg. Med. Chem. Lett. 21 June 1999, Vol. 9, pages 1667-1672, see entire document.	21-25, 27, 32, 33, 36, 48, 50-54, 56, 61, 62, 64, 75-77
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Y		1-12, 26, 28-31, 34, 35, 37-47, 49, 55, 57-60, 63, 65-74, 78
X	HARRIS et al. Rapid and General Profiling of Protease Specificity by using Combinatorial Fluorogenic Substrate Libraries. Proc. Natl. Acad. Sci. 05 July 2000, Vol. 97, No. 14, pages 7754-7759, see entire document.	21-25, 27, 32, 33, 36, 48, 50-54, 56, 61, 62, 64, 75-77
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Y		1-12, 26, 28-31, 34, 35, 37-47, 49, 55, 57-60, 63, 65-74, 78

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/49480

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-12 and 21-78
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/49480

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C12Q 1/00; G01N 33/53, 33/566; C12N 9/00; A01N 43/20; A61K 31/165; C07D 303/08

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/4, 7.1, 7.71, 7.72, 183; 436/501; 514/475, 624; 549/551, 552, 553

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, STN (Medline, CAPplus, BIOSIS, EMBASE, USPatfull)

Terms: cysteine, thiol, protease, hydrolase, inhibit, library, combinatorial, epoxide, ketone, sulfone, succinic. Structure search in Registry database.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-6, drawn to a system.

Group II, claim(s) 7-12, drawn to a compound.

Group III, claim(s) 13-15, drawn to a cell.

Group IV, claim(s) 16-20, drawn to a method for determining the presence of at least one active papain cysteine hydrolase target.

Group V, claim(s) 21-49, drawn to a probe.

Group VI, claim(s) 50-78, drawn to a probe library.

Group VII, claim(s) 79-106, drawn to a method of identifying or determining activity of a cysteine hydrolase.

Group VIII, claim(s) 107-126, drawn to a method of identifying an agent that modulates activity of a cysteine hydrolase.

Group IX, claim(s) 127-135, drawn to a kit.

The inventions listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

First, the technical feature that links the claims of Groups I – IV does not appear to be the same as that which links the claims of Groups V – IX. The technical feature that links the claims of Groups I – IV is the compound of structure A-L1-Hy-L2-E, while the technical feature that links the claims of Groups V – IX is the compound of structure A-L1-(aa1)i-(aa2)j-(aa3)k-(aa4)l-L2-E (where at least two aa are present). It is noted that Hy in claim 7 is defined as "a neutral hydrophobic amino acid". As stated above, the compound of A-L1-(aa1)i-(aa2)j-(aa3)k-(aa4)l-L2-E must contain at least two amino acids.

Furthermore, the claims of Groups I – IV lack unity because the claimed compounds are *known in the art* as disclosed by Inoue et al (US 5,935,939) and Tsubotani et al (WO 95/32954). These references are described below.

Inoue et al disclose piperazine-containing compounds that are cysteine protease inhibitors (see Abstract). Particularly, the compounds of Inoue et al read on those claimed containing an epoxide group (E) linked by a -C(O)- moiety (L2) to a hydrophobic amino acid group (Hy) where the piperazine moiety in the compounds of Inoue et al read on the claimed "moiety that provides a detectable signal or ligand" (A). See compounds of Inoue et al described in columns 2-3 of the reference and specifically exemplified compounds (such as those in Table 1-1).

Tsubotani et al disclose compounds that are protease inhibitors (see Abstract). Particularly, the compounds of Tsubotani et al read on those claimed containing an epoxide group (E) linked by a -C(O)- moiety (L2) to a amino acid group (Hy) which is preferably hydrophobic. The compounds of Tsubotani et al further contain a -CO-N(R2)R3 group; this group reads on the claimed "moiety that provides a detectable signal or ligand" (A). See compounds of

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Tsubotani et al described on pages 2-4 of the reference and specifically exemplified compounds (such as those on pages 29-30).

Moreover, see MPEP 1850: Unity of invention has to be considered in the first place only in relation to the independent claims in an international application and not the dependent claims. By "dependent" claim is meant a claim which contains all the features of another claim and is in the same category of claim as that other claim (the expression "category of claim" referring to the classification of claims according to the subject matter of the invention claimed, for example, product, process, use or apparatus or means, etc.).

Also see 37 CFR § 1.475 Unity of invention before the International Searching Authority, the International Preliminary Examining Authority and during the national stage, cited in part below (especially sections (c) and (d)).

(a) An international and a national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention"). Where a group of inventions is claimed in an application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

(b) An international or a national stage application containing claims to different categories of invention will be considered to have unity of invention if the claims are drawn only to one of the following combinations of categories:

A product and a process specially adapted for the manufacture of said product; or

A product and process of use of said product; or

A product, a process specially adapted for the manufacture of the said product, and a use of the said product; or

A process and an apparatus or means specifically designed for carrying out the said process; or

A product, a process specially adapted for the manufacture of the said product, and an apparatus or means specifically designed for carrying out the said process.

(c) If an application contains claims to more or less than one of the combinations of categories of invention set forth in paragraph (b) of this section, unity of invention might not be present.

(d) If multiple products, processes of manufacture or uses are claimed, the first invention of the category first mentioned in the claims of the application and the first recited invention of each of the other categories related thereto will be considered as the main invention in the claims, see PCT Article 17(3)(a) and § 1.476(c).

The instant international application contains multiple products and methods, where the feature that links the claims of "the first invention of the category first mentioned in the claims" is known in the art, as set forth above. Thus, the instant claims lack unity of invention. For these reasons, election under these rules is proper and required.

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